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Keywords: Helminth-microbiota interactions; Thoroughbred horses; cyathostomins; 16S rRNA sequencing; Adlercreuzia; Methanomicrobia; TM7.

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Order of Authors: Laura Peachey; Rebecca Molena; Timothy Jenkins; Angela Di Cesare; Donato Traversa; Jane Hodgkinson; Cinzia Cantacessi

Manuscript Region of Origin: UNITED KINGDOM

Abstract: A growing body of evidence, particularly in humans and rodents, supports the existence of a complex network of interactions occurring between gastrointestinal (GI) helminth parasites and the gut commensal bacteria, with substantial effects on both host immunity and metabolic potential. However, little is known of the fundamental biology of such interactions in other animal species. Nonetheless, given the considerable economic losses associated with GI parasites, particularly in livestock and equines, as well as the global threat of emerging anthelmintic resistance, further explorations of the complexities of host-helminth-microbiota interactions in these species are needed. This study characterises, for the first time, the composition of the equine bacterial commensal flora associated with the presence, in faecal samples, of low (Clow) - and high (Chigh) numbers of eggs of an important group of equine GI parasites (i.e. the cyathostomins), prior to and following anthelmintic treatment. High-throughput sequencing of microbial 16S rRNA amplicons and associated bioinformatics and statistical analyses of sequence data revealed strong clustering according to faecal egg counts (FEC) ($P = 0.003$). A trend towards increased populations of Methanomicrobia (class) and Dehalobacterium (genus) was observed in Clow in comparison to Chigh. Anthelmintic treatment in Chigh was associated with a significant reduction of the bacterial phylum TM7 14 days post-ivermectin administration, as well as a transient expansion of Adlercreuzia spp. at 2 days post-treatment. This study provides a first insight into the discovery of the intimate mechanisms governing host-parasite-microbiota interactions in equids, and sets a basis for the development of novel, biology-based intervention strategies against equine GI helminths based on the manipulation of the commensal gut flora.

UNIVERSITY OF CAMBRIDGE

21 November 2017

Dear Professor Slapeta,

REJOINDER:
R.E. Ms. No. IJPara17_387
Entitled 'The relationships between faecal egg counts and gut microbial composition in UK Thoroughbreds infected by cyathostomins'

We are very grateful for the reviewers' and editor's time and efforts in evaluating this manuscript. We thank the reviewers for their positive comments regarding the quality of our manuscript. The points raised by the reviewers are constructive and have contributed to enhance the manuscript. We have provided responses to individual points in the following rejoinder:

Editor's comment:
Both reviewers acknowledge the value and rigor of the submission. I would only add that you may consider including any data such as QIIME input files inc. metadata and OTUs into Mendeley Data https://data.mendeley.com/ to enable streamlined reproducibility. Mendeley Data will be directly linked to IJP site upon acceptance. If you do so include the DOI in the data availability section of your Materials & Methods

Our comment: The authors wish to thank the Editor for his kind evaluation. As suggested, raw and curated data have been deposited in the Mendeley database under doi:10.17632/g7chkjrp8f.1. Details have been included in the revised version of the manuscript.

Reviewer #1

This manuscript is an important contribution to the so-far neglected field of the equine microbiome and its relationship with the ubiquitous cyathostomins. This area is likely to provide fruitful in terms of advancing our understanding of basic gut biology, in horses and through a one health approach, in improving control of cyathostomins, including of acute larval cyathostominosis, and in providing improved treatments of colic, colitis and equine inflammatory bowel disease.

The authors have adopted an approach which does not require experimental infection, which is ethically laudable. This approach, however, will require confirmation in future studies from this and other groups in order to demonstrate reproducibility and consistency. Notably, the target group were pregnant broodmares, which in itself may have a bearing on the results.

Our comment: The authors thank Reviewer 1 for their kind comments regarding the overall quality and interest of this manuscript. We agree that due to the experimental approach, repetition of this study in different groups of horses is required to validate our results, as has been noted in the discussion (lines 467-470).

1) There are a few clarifications which the authors may wish to make in order to improve the reader experience. I note the samples were voided naturally. Were any precautions taken to avoid contamination from bacteria on the ground, such as taking samples from the inside of the mass only? Also, were the samples stored /transported anaerobically to preserve the detection of strict anaerobes?

Our comment: We can confirm that the samples were indeed taken from several points inside the faecal mass to avoid contamination from bacteria on the ground, and additionally, that the samples were snap frozen before transport and subsequent storage to preserve detection of bacterial taxa present at the time of voiding. Please see lines 136 and 141, respectively, for clarification of these points.

2) Is there any information on the stage/range of gestation stages of the broodmares in the study?

Our comment: All broodmares enrolled in our study were between months 5-8 of gestation at the start of the trial. This information has been added to the revised manuscript (lines 111-112).

3) The purpose of taking the control samples from non-pregnant animals could perhaps be better explained.

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4) I don't understand the percentages given for detection of each of the cyathostomin species - please clarify.

Our comment: These percentages represent the proportion of study participants which were positive for any given cyathostomin species. This information has been added to the revised manuscript (lines 215-216).

Reviewer #2

The manuscript is meritorious in terms of the quality, experimental design and methods. Interesting paper and it is worth publishing.

Our comment: We Reviewer 2 for their kind comments regarding the quality of our manuscript.

1) In the reference list, Nielsen et al 2010 is reported twice (see lines 690 and 697), delete that to line 697

Our comment: Addressed (line 686).

In conclusion, we consider that we have addressed each of the points raised by each of the reviewers. We agree with the points raised, and modified the text accordingly. The criticisms, suggestions, statements and comments have led to an enhanced manuscript, which we consider to meet the standard for publication in *International Journal of Parasitology*.

Yours sincerely,
Cinzia Cantacessi
On behalf of all authors.

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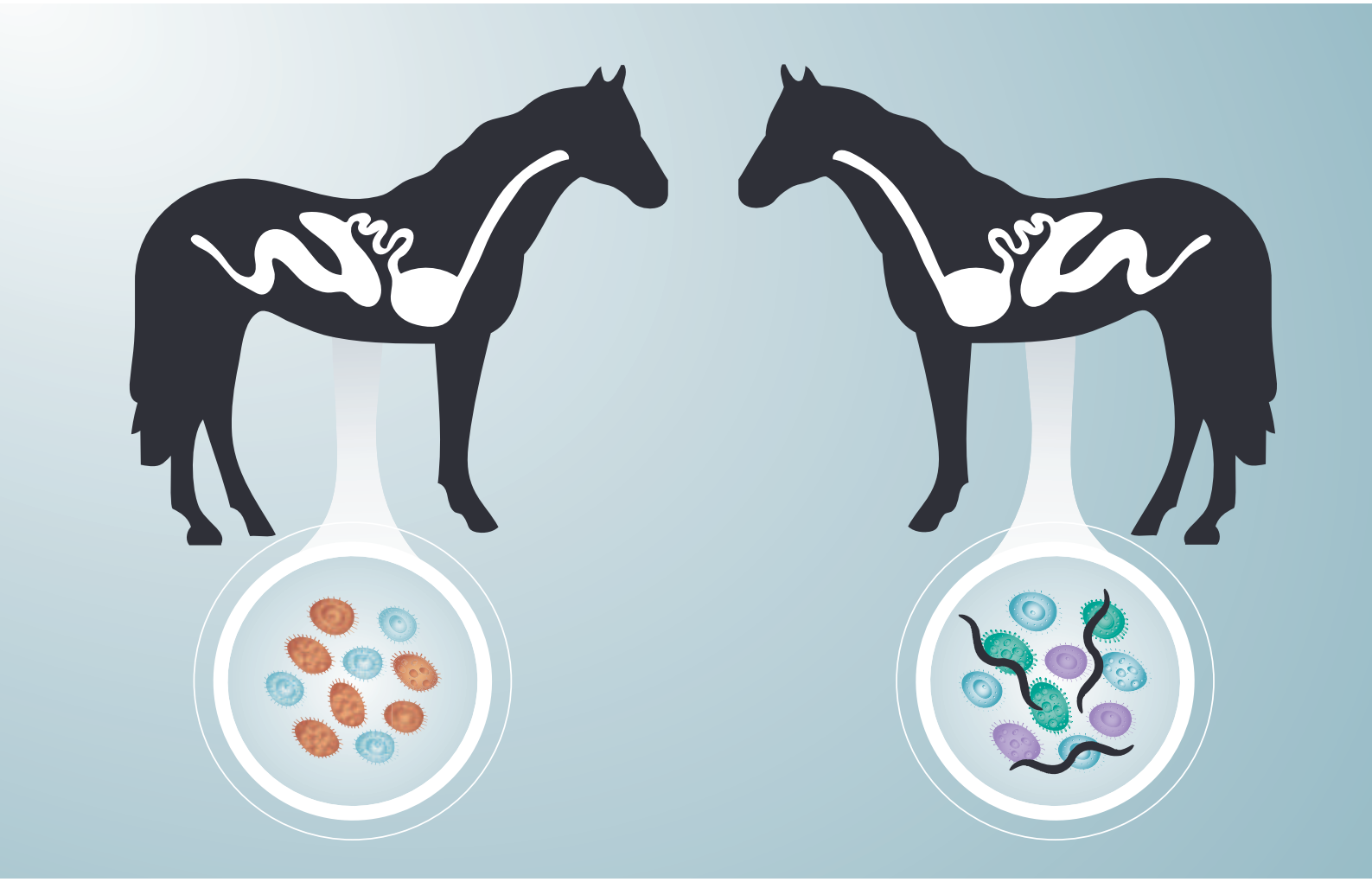
*Graphical Abstract (for review)



Highlights

- We studied qualitative and quantitative differences in faecal microbial profilings between groups of Thoroughbred horses with high- (C*high*) and low (C*low*) faecal egg counts of cyathostomin parasites, pre- and post-anthelmintic treatment.

- A trend towards increased populations of Methanomicrobia (class) and *Dehalobacterium* (genus) was observed in C*low* in comparison to C*high*.

- Anthelmintic treatment in C*high* was associated with a significant reduction of the bacterial phylum TM7 14 days post-ivermectin administration, as well as a transient expansion of *Adlercreuzia* spp. at 2 days post-treatment.

- This study sets a basis for the development of novel, biology-based intervention strategies against equine GI helminths based on the manipulation of the commensal gut flora.

The relationships between faecal egg counts and gut microbial composition in UK Thoroughbreds infected by cyathostomins

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Abstract

A growing body of evidence, particularly in humans and rodents, supports the existence of a complex network of interactions occurring between gastrointestinal (GI) helminth parasites and the gut commensal bacteria, with substantial effects on both host immunity and metabolic potential. However, little is known of the fundamental biology of such interactions in other animal species. Nonetheless, given the considerable economic losses associated with GI parasites, particularly in livestock and equines, as well as the global threat of emerging anthelmintic resistance, further explorations of the complexities of host-helminth-microbiota interactions in these species are needed. This study characterises, for the first time, the composition of the equine bacterial commensal flora associated with the presence, in faecal samples, of low (C*low*) - and high (C*high*) numbers of eggs of an important group of equine GI parasites (i.e. the cyathostomins), prior to and following anthelmintic treatment. High-throughput sequencing of microbial 16S rRNA amplicons and associated bioinformatics and statistical analyses of sequence data revealed strong clustering according to faecal egg counts (FEC) (P = 0.003). A trend towards increased populations of Methanomicrobia (class) and *Dehalobacterium* (genus) was observed in C*low* in comparison to C*high*. Anthelmintic treatment in C*high* was associated with a significant reduction of the bacterial phylum TM7 14 days post-ivermectin administration, as well as a transient expansion of *Adlercreuzia* spp. at 2 days post-treatment. This study provides a first insight into the discovery of the intimate mechanisms governing host-parasite-microbiota interactions in equids, and sets a basis for the development of novel, biology-based intervention strategies against equine GI helminths based on the manipulation of the commensal gut flora.

Key words: Helminth-microbiota interactions, Thoroughbred horses, cyathostomins, 16S rRNA sequencing, *Adlercreuzia*, Methanomicrobia, TM7.

1. **Introduction**

Cyathostomins are amongst the most important intestinal nematodes of horses globally (Love et al., 1999; Matthews, 2011; Stratford et al., 2011) with reported prevalence rates as high as 89-100% in equine herds (Mfitilodze and Hutchinson, 1990; Collobert-Laugier et al., 2002; Hinney et al., 2011; Morariu et al., 2016). Clinical signs of cyathostomin infection range from non-specific weight loss to colic and colitis caused by mass emergence of larvae from the large intestinal wall (= larval cyathostominosis), which may prove fatal (Uhlinger, 1991; Murphy and Love, 1997; Lyons et al., 2000; Peregrine et al., 2006). Thoroughbred (TB) young-stock kept in herds are at high risk of developing serious complications of infection, and hence the implementation of effective strategies for parasite control is a top priority for the TB equine industry. Control of cyathostomin infections has traditionally relied on the regular administration of chemotherapeutics (i.e. anthelmintics); however, the frequent and uncontrolled use of these compounds has led to the global emergence of resistant populations of parasites (Nielsen et al., 2014; Peregrine et al., 2014). In particular, foci of multi-drug resistance have been recently reported in UK TB stud farms (Relf et al., 2014). This observation, coupled with the lack of novel anthelmintic compounds licenced for use in equids, represents a 'Damocle's sword' for the UK (and global) equine industry. Therefore, alternative strategies for parasite control are urgently needed; in order to support the discovery of such strategies, a deeper understanding of the complex interactions occurring at the host-parasite interface, particularly at the site/s of infection (i.e. the gut), is required.

While a multitude of factors are responsible for the host-parasite interactions which determine infection outcome, increasing attention is being paid to the complex interplay between GI parasites and the host commensal gut microbiota (Bancroft et al., 2012; Glendinning et al., 2014). Indeed, recent studies have reported significant fluctuations in the composition of the vertebrate gut microbiota associated with helminth infections, that were

accompanied with shifts in both systemic and local immunity (Bancroft et al., 2012; Leung and Loke, 2013; Fricke et al., 2015; Houlden et al., 2015; Cattadori et al., 2016; Gause and Maizels, 2016). However, thus far, knowledge of helminth–microbiota cross-talk relies heavily on studies conducted in humans and/or rodent models of infection and disease (Walk et al., 2010; Rausch et al., 2013; Cantacessi et al., 2014; Lee et al., 2014; Reynolds et al., 2014a; Fricke et al., 2015; Giacomin et al., 2015; Holm et al., 2015; Houlden et al., 2015; Kreisinger et al., 2015; McKenney et al., 2015; Cattadori et al., 2016; Giacomin et al., 2016). Indeed, while only a handful of studies have characterised the composition of the gut microbiota of veterinary species infected by GI helminths thus far (Li et al., 2011; Li et al., 2012; Slapeta et al., 2015; Wu et al., 2012; Duarte et al., 2016; Li et al., 2016; reviewed by Peachey et al., 2017), no data is currently available on the effects of infections by GI helminths, such as cyathostomins, on the composition of the equine commensal flora. Aquiring this fundamental knowledge will be key to the development of novel holistic approaches to equid parasite control, aimed at improving host reponses to infections. In this study, we characterise, for the first time, the gut microbial profiles of a cohort of UK TB broodmares with low- and high-numbers of cyathostomin eggs in faeces (as determined by faecal egg counts analysis [FEC]), and examine the effects that administration of a commonly used anthelmintic, i.e. ivermectin, exerts on the overall composition of the gut microbiota as well as relative abundances of individual microbial species.

2. Materials and Methods

2.1. Ethics statement

This study was approved and carried out in strict accordance and compliance with the guidelines of the Institutional Ethical Review Committee, Department of Veterinary Medicine, University of Cambridge, UK (Research Project No. CR190). Written informed consent was obtained from the stud farm from which study samples were collected.

2.2. Sampling and diagnostic procedures

For this study, a cohort of TB broodmares was recruited from a stud farm in the East of England, UK. The stud hosts a group of ~130 pregnant broodmares each year, which are kept at pasture in groups of 2-8 across a 1200-acre farm. All broodmares are subjected to targeted anthelmintic treatments (ivermectin and moxidectin), based on FEC measurements at three monthly intervals. In addition, praziquantel is administered to each broodmare three times a year for tapeworm control, whilst a single moxidectin treatment is administered in late November for control of encysted cyathostomin larvae. Samples used in this study were collected in September-October 2016; all broodmares had received ivermectin and praziquantel in May and August 2016, respectively. A total of 117 TB pregnant broodmares, between 5 and 8 months of gestation at the time of sampling, were screened for infections by cyathostomins. Briefly, individual faecal samples were collected on three consecutive days over a seven-day period; aliquots of each sample were subjected to FEC analysis using a centrifugal floatation technique sensitive to one egg per gram (e.p.g.) (Christie and Jackson, 1982), and to screening for infections by the common equine cestode *Anoplocephala perfoliata* using a double sugar flotation technique (Rehbein et al., 2011). Upon observation of strongyle eggs during FEC analysis, the remaining faecal aliquots were subjected to larval culture to allow for subsequent identification of infecting nematode species using an established Reverse Line Blot (RLB) hybridisation method (Traversa et al., 2007; Cwiklinski et al., 2012). Briefly, genomic DNA was extracted from individual third stage larvae (L3s)

harvested from each larval culture, and the intergenic spacer (IGS) region was amplified by nested PCR using conserved biotin labelled primers (Traversa et al., 2007). The PCR products were then incubated with biodyne C membrane bound specific DNA probes for 21 different cyathostomin species (Cwiklinski et al., 2012), incubated with extravidin peroxidase and visualised using x-ray film. Horses were recruited in our study if they satisfied the following criteria: (i) FEC of >100 e.p.g. (= C*high*) or <10 e.p.g. (C*low*) in three consecutive samples collected over a seven-day period; (ii) matched by approximate age and paddock; (iii) negative for co-infections with other gastrointestinal (GI) helminths; (iv) no antibiotic treatment for at least two months prior to sampling; and (v) no previous anthelmintic treatment other than praziquantel for at least four months prior to sampling. Horses enrolled were kept at pasture for the duration of the study and fed 1kg of custom concentrate mix daily.

2.3. Anthelmintic treatment

Individual, naturally voided, faecal samples were collected from the centre of the faecal mass from C*high* and C*low* animals, as well as from three non-pregnant broodmares on day 0 (D0). Then, an anthelmintic treatment (Eqvalan: ivermectin 0.2 mg/kg) was immediately administered to each animal. Sampling was repeated as above at day 2 (D2) and day 14 (D14) post-treatment (p.t.). A 100g aliquot of each faecal sample was snap frozen, transported to the laboratory and stored at -20 ºC within 2h of collection, prior to genomic DNA extraction and sequencing of the bacterial 16S rRNA gene (see below), while the remainder was kept fresh and subjected to FEC analysis as described above.

2.4. High-throughput 16S rRNA sequencing

Genomic DNA was extracted from each faecal sample, as well as from five negative 'blank'

(= no DNA) controls, using the PowerSoil® DNA Isolation Kit (Qiagen, Carlsbad, CA, USA), according to the manufacturers' instructions. High-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed on an Illumina MiSeq platform according to the manufacturers' protocols with minor adjustments. Briefly, the V3-V4 region was PCR amplified using universal primers (Forward, 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′; Reverse, 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) (Klindworth et al., 2013), that contained the Illumina adapter over-hang nucleotide sequences, using the NEBNext® Q5® Hot Start HiFi DNA polymerase (New England Biolabs® Inc, Massachusetts, USA) and the following thermocycling protocol: 98 °C/2 min, followed by 20 cycles of 98 °C/15 s, 63 °C/30 s, and 72 °C/30 s, and a final elongation step of 72 °C/5 min. Amplicons were purified using AMPure XP PCR Purification beads (Beckman Coulter, Brea, California, USA), and the NEBNext hot start high-fidelity DNA polymerase was used for the index PCR with Nextera XT index primers (Illumina, San Diego, California, USA) according to the following thermocycling protocol: 98 °C/30 s, 8 cycles of 98 °C/10 s, 65 °C/75 s and at 65 °C/5 min. The indexed samples were purified using AMPure XP beads, quantified using the Qubit Quant-iTTM dsDNA Broad-Range Assay Kit (Life Technologies, Carlsbad, California, USA), and equal quantities from each sample pooled. The resulting pooled library was quantified using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs® Inc) and sequenced on an Illumina MiSeq platform using the v3 chemistry (301 bp paired-end reads).

2.5. Bioinformatics analyses

Raw paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/), and reads were joined using the

Quantitative Insights Into Microbial Ecology (QIIME) software suite (version 1.9.0) (Caporaso et al., 2010). Successfully joined sequences were quality filtered in QIIME using the 'usearch_qf' script with default settings. Then, sequences were clustered into Operational Taxonomic Units (OTUs) on the basis of similarity to known bacterial sequences available in the Greengenes database (v13.8; http://greengenes.secondgenome.com/; 97% sequence similarity cut-off) using the UCLUST software; sequences that could not be matched to references in the Greengenes database were clustered *de novo* based on pair-wise sequence identity (97% sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU. Then, representative sequences were assigned to taxonomy using the UCLUST software. OTUs assigned to sequences obtained from the no-DNA control samples, as well as singleton OTUs, were removed prior to downstream analysis. Normalisation was carried out by generating a subsampled OTU table by random sampling (without replacement) of the input OTU table using an implementation of the Mersenne twister algorithm (http://www.numpy.org). Cumulative-sum scaling (CSS) and log2 transformation were applied to account for the non-normal distribution of taxonomic counts data. Statistical analyses were executed using the Calypso software (cgenome.net/calypso/); samples were clustered using supervised Canonical Correlation Analysis including FEC (C*high*:C*low*) and time-point (D0, D2 and D14 p.t.) as explanatory variables. Differences in bacterial alpha diversity (Shannon diversity) between groups were evaluated using paired t-test or ANOVA, depending on number of groups for comparison. Beta diversity was calculated using weighted UniFrac distances and based on the matrices, differences in beta diversity were calculated using Permutational Analysis of Multivariate Dispersions (PERMDISP) through the 'betadisper' function (Anderson et al., 2006). Differences in the composition of the faecal microbiota between groups were assessed using the LEfSe (Linear discriminant analysis Effect Size) workflow (Segata et al., 2011), by

assigning FEC/timepoint 'groupings' as comparison class. All statistical analyses were repeated on a sub-group of horses with FEC >200 e.p.g. (n=8) and 0 e.p.g. (n=7), hereafter referred to as 'C*200*' and 'C*0'*, respectively.

3. Results

3.1 Prevalence of cyathostomin infection and group selection

Of 117 broodmares examined for cyathostomin infection, 36 matched the criteria outlined in section *2.2*. and were therefore enrolled in this study. Of these, 18 broodmares had an average FEC of ≥ 100 e.p.g. (range 100 - 418), and were thus enrolled as C*high* (Table 1). Amongst these, 8 were defined as C*200* based on average FEC of ≥ 200 e.p.g. (Table 1). Conversely, 18 broodmares with FEC of ≤ 10 e.p.g. (range 0 - 10) were enrolled as C*low* and, of these, 7 could be defined as C*0* (Table 1). While no known effect size estimates for changes in microbiota due to parasitism in horses are currently available, data from previous studies in other host:helminth systems (effect size: 1.5; cf. Giacomin et al., 2015, 2016) provided us with 84% power to detect changes in gut microbial composition between groups (n=18 in each C*high* and C*low* group) using canonical analysis of principal coordinates.

Larval culture followed by species identification *via* RLB revealed infections by the following cyathostomin species: *Cyathostomum (Cya.) catinatum* (88%), *Cylicostephanus (Cys.) longibursatus* (71%), *Cylicocyclus (Cyc.) nassatus* (59%), *Coronocyclus coronatus* (41%), *Cyc. calicatus* (41%), *Cyc. radiatus* (29%), *Cys. goldi* (24%), *Cys. leptostomus* (18%), *Cya. pateratum* (18%), *Cyc. ashworthi* (18%) and *Cyc. insigne* (12%). FEC analysis performed on samples collected at D2 and D14 p.t. showed FEC reduction rates (FECR) of > 95% in all treated animals (Table 1).

223 *3.2 Microbiota profiling*

224 A total of 39,461,550 raw paired-end reads were generated from DNA faecal extracts of
225 C*high* and C*low* broodmares and subjected to further processing. Following primer trimming,
226 joining of paired-end reads, filtering of low-quality sequences and removal of 'contaminant'
227 and singleton OTUs, a total of 8,077,490 (mean = 73,423 ± 3,610) high quality sequences
228 were retained for further bioinformatics analysis (not shown). Raw and curated sequence data
229 generated in this study are available from Mendeley Data at doi:10.17632/g7chkjrp8f.1. The
230 rarefaction curves generated following in-silico subtraction of low-quality and contaminant
231 sequences indicated that the vast majority of faecal bacterial communities were represented
232 in the remaining sequence data, thus allowing us to undertake further analyses. These
233 sequences were assigned to 95,286 OTUs and 15 bacterial phyla, respectively. The phyla
234 Bacteroidetes (39.9%) and Firmicutes (34.0%) were predominant in all samples, followed by
235 the phyla Verrucomicrobia (12.0%), Spirochaetes (3.9%), Fibrobacteres (2.4%),
236 Cyanobacteria (1%), Proteobacteria (0.9%), Euryarcheota (0.4%), Tenericutes (0.4%), TM7
237 (0.3%), Actinobacteria (0.3%), Lentispheraerae (0.3%), Synergistetes (0.2%), WPS-2 (0.2%)
238 and Planctomycetes (0.1%) (Fig. 1), while 3.4% of OTUs could not be assigned to any
239 bacterial group. Predominant sub-taxa were Bacteroidia (class), Bacteroidales (order) and
240 Bacteroidales (family) within the phylum Bacteroidetes, and Clostridia (class), Clostridiales
241 (order) and Ruminococcae (family) within the Firmicutes (Fig. 1). Two samples, LVN1 and
242 HS2, differed markedly in the relative proportions of the two most abundant phyla,
243 Bacteroidetes and Firmicutes, when compared with samples from other broodmares (Fig. 1),
244 likely indicating dysbiosis. Therefore, in order to reduce biases due to these potential
245 'outliers', these samples were excluded from further statistical analyses (Fig. 1).
246

Figure 1: Bar charts depicting the relative abundances of faecal bacterial phyla from broodmares with faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (= C*high*) and <10 e.p.g. (= C*low*), according to sampling time point (i.e. pre-anthelmintic treatment [D0], and 2 and 14 days post-treatment [D2 and D14, respectively], and in non-pregnant controls (NPC). Samples from broodmares with FEC >200 e.p.g. (*C200*) and 0 (*C0*) are shown in red.

3.3. Differences in microbial composition between Chigh and Clow, and pre- and post-anthelmintic treatment

Microbial community profiles of each sample were grouped by hierarchical clustering and ordinated by supervised CCA. Using these methods, a significant association was observed between microbial composition and FEC (C*high versus* C*low*) (P = 0.003), while clustering according to time point pre- and post- anthelmintic treatment (D0 *versus* D14) did not reach statistical significance (P = 0.686) (Fig. 2a). CCA of C*200 versus* C*0* led to a clear separation according to FEC (P = 0.001), whilst the effect of anthelmintic treatment remained insignificant (P = 0.811) (Fig. 2b).

Figure 2: The microbial composition of faecal samples ordered by supervised canonical correspondence analysis (CCA) from broodmares with (a) faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (= C*high*) and <10 e.p.g. (= C*low*), pre-anthelmintic treatment [D0] and at 14 days post-treatment [D14], (b) with FEC >200 e.p.g. (*C200*) and 0 (*C0*) at D0 and D14.

No significant differences in OTU alpha diversity (Shannon Index) were recorded between C*high* and C*low*, or between samples collected at D0, D2 and D14 p.t. (Fig.3 a,b,c). A trend

towards increased alpha diversity in C*high versus* C*low* at all time-points was observed (P = 0.087) (Fig 3a). This trend was also observed when C*200* samples were compared to C*0* at D0, despite smaller group sizes (P = 0.102) (Fig. 3c). No significant differences in beta diversity, as measured by PERMDISP, were observed between groups (Fig. 4).

Figure 3: Shannon diversity charts comparing faecal microbial alpha diversity of broodmares (a) with faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (= C*high*) and <10 e.p.g. (= C*low*) at all time points (i.e. pre-anthelmintic treatment [D0] and 2 and 14 days post-treatment [D2 and D14, respectively]), (b) C*high* and C*low* at D0 only, (c) with FEC >200 e.p.g. (C*200*) and 0 (C*0*) at D0 only, and (d) C*high* and (e) C*low* at D0, D2 and D14.

Figure 4: Permutational Analysis of Multivariate Dispersions (PERMDISP) plots comparing the faecal microbial beta diversity of broodmares (a) with faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (C*high*) and <10 e.p.g. (C*low*) at all time points (i.e. pre-anthelmintic treatment [D0] and 2 and 14 days post-treatment [D2 and D14, respectively]), (b) C*high* and C*low* at D0 only, (c) with FEC >200 e.p.g. (C*200*) and 0 (C*0*) at D0 only, and (d) C*high* and (e) *Clow* at D0, D2 and D14.

Differences in abundance of individual taxa at the phylum, class, order, family, genus and species level were detected between C*high* and C*low* samples, as well as between samples collected at D0, and D2 and D14 p.t. (Fig. 5). Samples from C*low* at D0 (pre-treatment) were characterised by an increased abundance of Methanobacteria (class), *Dehalobacterium* (genus) and unclassified *Dehalobacterium* and *Ruminococcus* (species) compared with samples from C*high* (Fig. 5a). The same taxa were increased in C*0* compared with C*200*,

297 with the addition of methanogens of the family Methanocorpusculacaea belonging to order
298 Methanomicrobiales, class Methanobacteria, phylum Euryarchaeota; order Endomicrobiales,
299 phylum Elusimicrobia; Rickettsiales (order, family, genus, species); family Bacteroidaceae,
300 genus BF311 and species RFN20 (Fig. 5b). The taxa GMD14H09 (order, family, genus,
301 species) of the phylum Proteobacteria were increased in samples from C*200* compared with
302 C*0* (Fig. 5b). Anthelmintic treatment in C*high* was accompanied by a decrease in the phylum
303 TM7 at D14, when compared with pre-treatment samples (Fig. 5c). Additionally, the taxa
304 *Adlercreutzia* and R445B were increased at D2 and D14, respectively, compared with D0
305 samples (Fig. 5c). In C*low*, treatment was also associated with an increase in R445B (family,
306 genus, species) at D14 (Fig. 5d).
307
308 **Figure 5: Linear discriminant analysis Effect Size (LEfSe) bar charts depicting**
309 **differences in abundance of individual bacterial taxa at the phylum, class, order, family,**
310 **genus and species level in faecal samples from broodmares (a) with faecal egg counts**
311 **(FEC) >100 eggs per gram (e.p.g.) (C*high*) and <10 e.p.g. (C*low*), (b) with FEC >200**
312 **e.p.g. (*C200*) and 0 (*C0*) at D0, and in (c) *Chigh* and (d) C*low* according to sampling time**
313 **point (i.e. pre-anthelmintic treatment [D0] and 2 and 14 days post-treatment [D2 and**
314 **D14, respectively]).**
315
316 **4. Discussion**
317 This study is the first to report a significant association between numbers of cyathostomin
318 eggs in faecal samples from UK Thoroughbreds and the composition of the host gut
319 microbiota. A particularly significant shift in microbial profiles was observed when the faecal
320 bacterial populations of a group of broodmares with FEC of >200 e.p.g. were compared with
321 those with observed FEC of 0. These data are consistent with observations from published

studies in both humans and other veterinary species, including rodent models of infection and disease (Lee et al., 2014; Holm et al., 2015; Houlden et al., 2015; McKenney et al., 2015; Duarte et al., 2016; Li et al., 2016). In addition, the administration of a routinely used anthelmintic (i.e. ivermectin) to both C*high* and C*low* resulted in further progressive changes of the microbial profiling of treated horses. While such changes did not reach statistical significance when analysed using a multivariate model, this trend suggests that parasite-associated modifications in gut microbiota may be transient, and dependent on the presence of live infections, a hypothesis which requires thorough testing.

Overall, the bacterial phyla identified in this study were consistent between groups of animals enrolled in this study; this observation differs from results from previous studies that had reported significant variability in faecal microbial profiling between horses, largely related to variations in diet and age, and presence of underlying diseases (Costa et al., 2012; Daly et al., 2012; Steelman et al., 2012; O' Donnell et al., 2013; Dougal et al., 2014; Fernandes et al., 2014; Weese et al., 2014). Thus, our finding likely indicates that the impact of such confounding factors was successfully minimised by our study design, and that the recorded differences in microbial composition were indeed associated to parasite infections. Bacteria belonging to the phylum Bacteroidetes were predominant in animals examined in our study; conversely, other investigations had reported Firmicutes as being the most prevalent phylum in the horse gut flora (Costa et al., 2012; Shepherd et al., 2012; Dougal et al., 2014; Fernandes et al., 2014; Weese et al., 2014; Costa et al., 2015a; Costa et al., 2015b; Proudman et al., 2015). Dietary differences between horse cohorts enrolled in this and previous studies are likely to be responsible for this discrepancy (cf. Daly et al., 2012; Fernandes et al., 2014).

346 Overall, a trend towards increased microbial alpha diversity, i.e. the number of different
347 OTUs in each sample [‘richness’] and their relative abundance [= ‘evenness’] (Tuomisto,
348 2010), was observed in samples from C*high* compared with those from C*low* at D0 (pre-
349 anthelmintic treatment) and in C*200 versus* C*0*, although these differences did not reach
350 statistical significance. Nevertheless, this observation is supported by the results of a number
351 of previous studies in other host:helminth systems, in which the establishment of parasite
352 infections was associated with an overall increase in alpha diversity of the gut microbiota
353 (Broadhurst et al., 2012; Lee et al., 2014; Giacomin et al., 2015; Giacomin et al., 2016).
354 Given that a number of inflammatory GI and/or systemic diseases are accompanied by a
355 reduced alpha diversity (Manichanh et al., 2006; Sepehri et al., 2007; Abrahamsson et al.,
356 2012, 2014), the increase in GI microbial diversity observed in the presence of helminth
357 infections has been hypothesized to represent one of the possible mechanisms by which
358 parasites suppress host inflammatory responses, thus ensuring their long-term survival in the
359 host gut (Bancroft et al., 2012; Glendinning et al., 2014). Therefore, the trends towards
360 increased alpha diversity observed in the faecal microbiota of horses moderately infected by
361 cyathostomins may also result from an increase in gut homeostasis promoted by the parasite.
362 Future studies evaluating the prevalence and incidence of equine inflammatory diseases (e.g.
363 inflammatory bowel disease and recurrent airway obstruction) in the presence or absence of
364 parasite infections could represent significant first steps in this area of research.
365
366 In addition to global microbial diversity, significant variations in the abundance of specific
367 bacterial taxa were observed between groups. In particular, a higher abundance of
368 microorganisms belonging to the class Methanomicrobia was observed in C*low* (D0) when
369 compared to C*high*. This difference was exacerbated in C*200 versus* C*0*, with further
370 significant increases in methanogens belonging to class Methanomicrobia recorded in C*0*,

thus suggesting a negative correlation between methanogen abundance and FEC. Methanomicrobia belong to the phylum Euryarchaeota, kingdom Archaea and are phylogenetically distinct from bacteria and eukaryotes, although they retain the prokaryote 16S rRNA gene (Woese and Gupta, 1981; Winker and Woese, 1991). Particularly in ruminants, the role of the Archaeal methanogens in the digestion of fibre has been well documented (St-Pierre et al., 2015). In equids, little is known of the functional diversity of methanogens; however, consistent with our findings, a recent study reported Methanomicrobiales as being predominant in the horse gut (Lwin and Matsui, 2014). The underlying mechanisms by which GI helminths may be promoting a reduction in populations of methanogens are yet unclear. Similarly to hypotheses formulated for other host:helminth systems, cyathostomins may prevent expansion of methanogens directly, e.g. *via* their excretory secretory products, or indirectly *via* parasite-induced changes in mucosal immunity (reviewed by Peachey et al., 2017). Alternatively, a high abundance of methanogens prior to helminth infections may bias host immune responses against cyathostomins, thus resulting in the observed low (or absent) parasite burdens. Interestingly, some methanogens (i.e. *Methanosphaera stadtmanae*) have been shown to regulate Th17 responses in mice (Blais Lecours et al., 2011; Bernatchez et al., 2017); in turn, these responses have been linked to the ability of mice to clear experimental infections by *Heligmosomoides polygyrus* (Reynolds et al., 2014b). Mechanistic studies aimed to evaluate the effects of expanding populations of gut methanogens on host mucosal responses and, in turn, GI helminth establishment, may help elucidating these interactions.

An increased abundance of Methanomicrobia in C*low* and C*0* may also be linked to other environmental factors that are simultaneously responsible for the low FEC observed. An example is represented by the horse grazing behaviour; indeed, it is known that some

individuals within a herd favour less nutritional swards of grass in order to avoid faecal contamination (Hutchings et al., 2000). In turn, as animal faeces often act as fertilisers, as a result, individuals favouring nutritious grass are exposed to higher numbers of infective larvae. Grazing different swards of grass may also impact on dietary fibre levels, and thus on gut methanogen populations, as observed in ruminants (McAllister et al., 1996). In horses, dietary factors have also been associated with changes in abundance of Methanomicrobia; for example, *Methanocorpusculum* archaea were observed at a median of 17.7% in horses fed a forage-grain diet, and at a median of 31.9% in horses maintained on pasture (Fernandes et al., 2014). Differences in grazing behaviour between individuals may also be accountable for the increased abundance of bacteria of the phylum Elusimicrobia in C*0* versus C*200* as these taxa are primarily a component of termite hind-gut microbiota (Gómez and González-Megías, 2007; van Klink et al., 2015; Mikaelyan et al., 2017). Experimental cyathostomin infections of stabled horses may eliminate the effect of grazing behaviour on gut microbial profiles, although ethical concerns may prevent the execution of such a study in the future.

In contrast to uninfected horses, the faecal microbial profiles of C*200* were characterised by an increased abundance of GMD14H09, phylum Proteobacteria, class Deltaproteobacteria. Increases in Proteobacteria abundance have repeatedly been reported in association with helminth infections, e.g. in mice infected by *Trichuris muris* and *H. polygyrus*, pigs infected by *T. suis*, and rabbits infected by *Trichostrongylus retortaeformis* (Li et al., 2012; Holm et al., 2015; Cattadori et al., 2016). Proteobacteria are known increase in the presence of GI inflammation (Shin et al., 2015); hence, the expansion of populations of Proteobacteria in the faecal microbiota of horses with higher infection burdens may be indicative of an inflammatory status of the intestinal tract of these horses at the time of sampling.

One of the objectives of this study was to assess the impact of anthelmintic treatment on the faecal microbial profiling of cyathostomin-infected horses. In particular, ivermectin administration to C*high* was followed by a significant decrease in populations of the phylum TM7 at D14. Since the relative abundance of TM7 remained unchanged following ivermectin administration in C*low*, it is tempting to speculate that a mutualistic association may exist between TM7 and cyathostomins, whereby each promote establishment of the other, similarly to the mutual relationship described for Lactobacillacaeae and *H. polygyrus* (Reynolds et al., 2014a). Bacteria belonging to the phylum TM7 are obligate epibionts of *Actinomyces* spp. (He et al., 2015), and are thus uncultivable. While TM7 have not previously been linked to GI helminth infections, this phylum of bacteria has been associated with mucosal inflammatory disease in humans (Kuehbacher et al., 2008). Interestingly, TM7 isolates have been shown to repress the induction of TNF-α production in macrophages infected by *Actinomyces odontolyticus*, thus suggesting a potential immune suppressive activity (He et al., 2015); hence, TM7 may promote the establishment of cyathostomins by suppressing effective anti-parasite immune responses. Furthermore, an increase of the taxa *Adlercreutzia* (phylum actinobacteria) and R445B (phylum Lentisphearae) was observed in C*high* at D2 and D14, respectively. The latter was also increased in C*low* at D14, suggesting that this change was unrelated to cyathostomin removal. Bacteria of the genus *Adlercreutzia* produce the metabolite equol (Maruo et al., 2008), a known anti-inflammatory and vasodilator (Blay et al., 2010). Thus, it could be hypothesised that increases in populations of *Adlercreutzia* and its metabolites following ivermectin administration might contribute to the emergence of hypobiotic larval stages of cyathostomins (which is known to occur post-anthelmintic treatment; Lyons et al., 2000), via the suppression of effective mucosal immune responses. This hypothesis requires testing in controlled mechanistic experiments.

FEC are often utilised as proxy of parasite infection burdens; however, several investigations

446 have confuted this practice, as weak correlations have been detected between FEC and
447 parasite burdens in horses with >500 e.p.g. of faeces (Nielsen et al., 2010). While the FEC
448 cut-offs used in this study are indicative of differing infection burdens between groups, any
449 inference on the relationships between number of worms in the horse intestine and gut
450 microbial profiling must be taken with caution. Ethical considerations prevent us from
451 performing post-mortem total worm counts on experimentally infected horses; nevertheless,
452 in the future, it may be possible to establish unequivocal relationships between cyathostomin
453 infection burdens (including encysted larvae) and gut microbial profiling from samples
454 collected in abattoir.
455

456 Clearly, a complex network of host-parasite interactions, as well as environmental factors,
457 contribute to the findings reported in this study, and thus further work is needed to dis-
458 entangle the causality of these relationships. However, one key question that needs
459 addressing is whether differences in host immunity may be associated with significant
460 changes in gut microbial composition (and *vice versa*) and, if such is the case, whether the
461 horse gut microbiota could be manipulated to improve resistance to helminth infection.
462 Indeed, previous investigations in cattle and mice have reported that host genes encoding for
463 antimicrobial proteins are up-regulated in the mucosae of animals resistant to helminth
464 infection (D'Elia et al., 2009; Li et al., 2015). In addition, dietary supplementation with both
465 pro- (Bautista-Garfias et al., 1999; Bautista-Garfias et al., 2001; Martinez-Gomez et al.,
466 2009; Martinez-Gomez et al., 2011; Oliveira-Sequeira et al., 2014; El Temsahy et al., 2015)
467 and pre-biotics (Petkevicius et al., 2003; Petkevicius et al., 2004; Thomsen et al., 2005;
468 Petkevicius et al., 2007; Jensen et al., 2011), has led to significant reductions in worm
469 burdens in murine and swine helminth infection models, thus indicating that alterations to the
470 bacterial flora in the host GI tract may bias host immune responses against parasites. Further

characterisation of equine host mucosal responses and GI microbiota, in the presence or absence of helminth infection and accompanied by total enumeration of infecting parasites, is a key area of future research, as it may lead to the identification of microbial factors linked to host susceptibility.

5. Conclusion

Cyathostomin infection in horses was associated with global shifts in faecal microbial composition and diversity, in accordance with previous studies in other host: helminth systems, as well as significant changes in specific populations of gut bacteria. Such changes predominantly involved 'minor' phyla, thus suggesting that the equine 'core gut microbiota' remains unaltered in the presence of burdens of cyathostomins such as those observed in this study. Our findings also support the hypothesis that selected bacterial taxa, and/or their metabolites, may play roles in biasing the host immune response either for, e.g. TM7, or against, e.g. Methanomicrobia, cyathostomin infection in horses. These data pave the way for future mechanistic studies aimed to identify microbial factors linked to host susceptibility, and to manipulate the GI microbiota of horses (e.g. via dietary or probiotic interventions), in order to improve resistance to cyathostomins.

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Table 1: Faecal egg counts (FEC) recorded from C*high* (FEC >100 eggs per gram, e.p.g.) and C*low* (FEC <100 e.p.g.) broodmares enrolled in the study, as well as of non-pregnant controls (NPC), over three consecutive samplings performed pre-anthelmintic treatment [D0], as well as at 2 and 14 days post-treatment [D2, D14]. Horses with FEC of > 200 e.p.g. (C*200*) and 0 e.p.g. (C*0*), are indicated in bold.

Group	*Animal i.d.*	*Age (yrs)*	*Tapeworm FEC (e.p.g.)*	*Ascarid FEC (e.p.g.)*	*Mean consecutive strongyle FEC (e.p.g.) (±SE) D0*	*FEC D2 (e.p.g.)*	*FEC D14 (e.p.g.)*
C*high*							
	MA	6	0	0	123 (±19)	0	0
	CT	5	0	0	130 (±14)	0	0
	LE	10	0	0	113 (±17)	0	0
	PB	5	0	0	171 (±15)	0	0
	SC	4	0	0	101 (±8)	2 (±2)	0
	MSJ	6	0	0	100 (±6)	0	0
	WD	7	0	0	128 (±16)	0	0
	NS	8	0	0	120 (±11)	1 (±1)	0
	HY	7	0	0	150 (±14)	0	0
	RM	4	0	0	139 (±49)	0	0
	HS	**6**	**0**	**0**	**200 (±39)**	**1 (±1)**	**1 (±1)**
	HT	**6**	**0**	**0**	**228 (±22)**	**0**	**0**
	NO	**6**	**0**	**0**	**271 (±17)**	**0**	**1 (±1)**
	QM	**4**	**0**	**0**	**418 (±112)**	**0**	**0**
	SB	**8**	**0**	**0**	**206 (±37)**	**1 (±1)**	**1 (±1)**
	TC	**4**	**0**	**0**	**235 (±12)**	**0**	**1 (±1)**
	MQ	**6**	**0**	**0**	**228 (±22)**	**0**	**0**
	VR	**4**	**0**	**0**	**279 (±61)**	**1 (±1)**	**0**
C*low*							
	GL	4	0	0	10 (±2)	0	0
	DWD	8	0	0	0.3 (± 0.3)	0	0
	LVN	4	0	0	5 (±1)	0	0
	BB	7	0	0	1 (±1)	0	4 (±1)
	LF	5	0	0	10 (±2)	0	0
	IR	7	0	0	3 (±1)	0	0
	DDR	8	0	0	1 (±1)	0	0
	LAL	5	0	0	0.3 (±0.3)	0	0
	PT	7	0	0	0.3 (±0.3)	0	0
	PP	6	0	0	2 (±1)	0	0
	MG	5	0	0	0.5 (±0.3)	0	0
	SY	**8**	**0**	**0**	**0**	**0**	**0**
	BX	**16**	**0**	**0**	**0**	**0**	**0**
	MR	**10**	**0**	**0**	**0**	**0**	**0**
	DD		**0**	**0**	**0**	**0**	**0**
	ED	**8**	**0**	**0**	**0**	**0**	**0**
	EP	**5**	**0**	**0**	**0**	**0**	**0**
	SWC	**8**	**0**	**0**	**0**	**0**	**0**
NPC							
	BET	8	0	0	412 (±45)	-	-

BL	12	0	0	22 (±1)	-	-
ST	10	0	0	0	-	-

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The relationships between faecal egg counts and gut microbial composition in UK Thoroughbreds infected by cyathostomins

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Abstract

A growing body of evidence, particularly in humans and rodents, supports the existence of a complex network of interactions occurring between gastrointestinal (GI) helminth parasites and the gut commensal bacteria, with substantial effects on both host immunity and metabolic potential. However, little is known of the fundamental biology of such interactions in other animal species. Nonetheless, given the considerable economic losses associated with GI parasites, particularly in livestock and equines, as well as the global threat of emerging anthelmintic resistance, further explorations of the complexities of host-helminth-microbiota interactions in these species are needed. This study characterises, for the first time, the composition of the equine bacterial commensal flora associated with the presence, in faecal samples, of low (C*low*) - and high (C*high*) numbers of eggs of an important group of equine GI parasites (i.e. the cyathostomins), prior to and following anthelmintic treatment. High-throughput sequencing of microbial 16S rRNA amplicons and associated bioinformatics and statistical analyses of sequence data revealed strong clustering according to faecal egg counts (FEC) (P = 0.003). A trend towards increased populations of Methanomicrobia (class) and *Dehalobacterium* (genus) was observed in C*low* in comparison to C*high*. Anthelmintic treatment in C*high* was associated with a significant reduction of the bacterial phylum TM7 14 days post-ivermectin administration, as well as a transient expansion of *Adlercreuzia* spp. at 2 days post-treatment. This study provides a first insight into the discovery of the intimate mechanisms governing host-parasite-microbiota interactions in equids, and sets a basis for the development of novel, biology-based intervention strategies against equine GI helminths based on the manipulation of the commensal gut flora.

Key words: Helminth-microbiota interactions, Thoroughbred horses, cyathostomins, 16S rRNA sequencing, *Adlercreuzia*, Methanomicrobia, TM7.

1. Introduction

Cyathostomins are amongst the most important intestinal nematodes of horses globally (Love et al., 1999; Matthews, 2011; Stratford et al., 2011) with reported prevalence rates as high as 89-100% in equine herds (Mfitilodze and Hutchinson, 1990; Collobert-Laugier et al., 2002; Hinney et al., 2011; ~~Mfitilodze and Hutchinson, 1990;~~ Morariu et al., 2016). Clinical signs of cyathostomin infection range from non-specific weight loss to colic and colitis caused by mass emergence of larvae from the large intestinal wall (= larval cyathostominosis), which may prove fatal (Uhlinger, 1991; Murphy and Love, 1997; Lyons et al., 2000; Peregrine et al., 2006). Thoroughbred (TB) young-stock kept in herds are at high risk of developing serious complications of infection, and hence the implementation of effective strategies for parasite control is a top priority for the TB equine industry. ~~Traditionally, c~~Control of cyathostomin infections ~~relies~~ has traditionally relied on the regular administration of chemotherapeutics (i.e. anthelmintics); however, the frequent and uncontrolled use of these compounds has led to the global emergence of resistant populations of parasites (Nielsen et al., 2014; Peregrine et al., 2014). In particular, foci of multi-drug resistance have been recently reported in UK TB stud farms (Relf et al., 2014). This observation, coupled with the lack of novel anthelmintic compounds licenced for use in equids, represents a 'Damocle's sword' for the UK (and global) equine industry. Therefore, alternative strategies for parasite control are urgently needed; in order to support the discovery of such strategies, a deeper understanding of the complex interactions occurring at the host-parasite interface, particularly at the site/s of infection (i.e. the gut), is required.

While a multitude of factors are responsible for the host-parasite interactions which determine infection outcome, increasing attention is being paid to the complex interplay between GI parasites and the host commensal gut microbiota (Bancroft et al., 2012; Glendinning et al., 2014). Indeed, recent studies have reported significant fluctuations in the

composition of the vertebrate gut microbiota associated with helminth infections, that were accompanied with shifts in both systemic and local immunity (Bancroft et al., 2012; Leung and Loke, 2013; Fricke et al., 2015; Houlden et al., 2015; Cattadori et al., 2016; ~~Fricke et al., 2015;~~ Gause and Maizels, 2016~~; Houlden et al., 2015; Leung and Loke, 2013~~). However, thus far, knowledge of helminth–microbiota cross-talk relies heavily on studies conducted in humans and/or rodent models of infection and disease (Walk et al., 2010; Rausch et al., 2013; Cantacessi et al., 2014~~; Cattadori et al., 2016~~; Lee et al., 2014; Reynolds et al., 2014a; Fricke et al., 2015; Giacomin et al., 2015~~; Giacomin et al., 2016~~; Holm et al., 2015; Houlden et al., 2015; Kreisinger et al., 2015~~; Lee et al., 2014~~; McKenney et al., 2015~~; Rausch et al., 2013; Reynolds et al., 2014b; Walk et al., 2010~~; Cattadori et al., 2016; Giacomin et al., 2016). ~~In particular~~Indeed, while only ~~, while~~ a ~~few~~ handful of studies have characterised the composition of the gut microbiota of veterinary species infected by GI helminths thus far (~~Duarte et al., 2016;~~ Li et al., 2011; Li et al., 2012~~; Li et al., 2016~~; Slapeta et al., 2015; Wu et al., 2012; ~~Slapeta et al., 2015;~~ Duarte et al., 2016; Li et al., 2016; reviewed by Peachey et al., 2017). ~~,~~ no data is ~~thus~~ currently ~~far~~ available on the effects of infections by GI helminths, such as cyathostomins, on the composition of the equine commensal flora. Aquiring this fundamental knowledge will be key to the development of novel holistic approaches to equid parasite control, aimed at improving host reponses to infections. In this study, we characterise, for the first time, the gut microbial profiles of a cohort of UK TB broodmares with low- and high-numbers of cyathostomin eggs in faeces (as determined by faecal egg counts analysis [FEC]), and examine the effects that administration of a commonly used anthelmintic, i.e. ivermectin, exerts on the overall composition of the gut microbiota as well as relative abundances of individual microbial species.

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2. Materials and Methods

2.1. Ethics statement

This study was approved and carried out in strict accordance and compliance with the guidelines of the Institutional Ethical Review Committee, Department of Veterinary Medicine, University of Cambridge, UK (Research Project No. CR190). Written informed consent was obtained from the stud farm from which study samples were collected.

2.2. Sampling and diagnostic procedures

For this study, a cohort of TB broodmares was recruited from a stud farm in the East of England, UK. The stud hosts a group of ~130 pregnant broodmares each year, which are kept at pasture in groups of 2-8 across a 1200-acre farm. All broodmares are subjected to targeted anthelmintic treatments (ivermectin and moxidectin), based on FEC measurements at three monthly intervals. In addition, praziquantel is administered to each broodmare three times a year for tapeworm control, whilst a single moxidectin treatment is administered in late November for control of encysted cyathostomin larvae. Samples used in this study were collected in September-October 2016; all broodmares had received ivermectin and praziquantel in May and August 2016, respectively. A total of 117 TB pregnant broodmares, between 5 and 8 months of gestation at the time of sampling, were screened for infections by cyathostomins. Briefly, individual faecal samples were collected on three consecutive days over a seven-day period; aliquots of each sample were subjected to FEC analysis using a centrifugal floatation technique sensitive to one egg per gram (e.p.g.) (Christie and Jackson, 1982), and to screening for infections by the common equine cestode *Anoplocephala perfoliata* using a double sugar flotation technique (Rehbein et al., 2011). Upon observation of strongyle eggs during FEC analysis, the remaining faecal aliquots were subjected to larval

culture to allow for subsequent identification of infecting nematode species using an established Reverse Line Blot (RLB) hybridisation method (Traversa et al., 2007; Cwiklinski et al., 2012). Briefly, genomic DNA was extracted from individual third stage larvae (L3s) harvested from each larval culture, and the intergenic spacer (IGS) region was amplified by nested PCR using conserved biotin labelled primers (Traversa et al., 2007). The PCR products were then incubated with biodyne C membrane bound specific DNA probes for 21 different cyathostomin species (Cwiklinski et al., 2012), incubated with extravidin peroxidase and visualised using x-ray film. Horses were recruited in our study if they satisfied the following criteria: (i) FEC of >100 e.p.g. (= C*high*) or <10 e.p.g. (C*low*) in three consecutive samples collected over a seven-day period; (ii) matched by approximate age and paddock; (iii) negative for co-infections with other gastrointestinal (GI) helminths; (iv) no antibiotic treatment for at least two months prior to sampling; and (v) no previous anthelmintic treatment other than praziquantel for at least four months prior to sampling. Horses enrolled were kept at pasture for the duration of the study and fed 1kg of custom concentrate mix daily.

2.3. Anthelmintic treatment

Individual, naturally voided, faecal samples were collected from the centre of the faecal mass from C*high* and C*low* animals, as well as from three non-pregnant broodmares on day 0 (D0). Then, an anthelmintic treatment (Eqvalan: ivermectin 0.2 mg/kg) was immediately administered to each animal. Sampling was repeated as above at day 2 (D2) and day 14 (D14) post-treatment (p.t.). A~~A~~ 100g aliquot of each faecal sample was snap frozen, transported to the laboratory and stored at -20 ºC within 2h of collection, prior to genomic DNA extraction and sequencing of the bacterial 16S rRNA gene (see below), while the remainder was kept fresh~~. The remainder of each sample was~~ and subjected to FEC analysis as described above.

150

151 *2.4. High-throughput 16S rRNA sequencing*

152 Genomic DNA was extracted from each ~~each individual~~ faecal sample, as well as from five
153 negative 'blank' (= no DNA) controls, using the PowerSoil® DNA Isolation Kit (Qiagen,
154 Carlsbad, CA, USA), according to the manufacturers' instructions. High-throughput
155 sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was
156 performed on an Illumina MiSeq platform according to the manufacturers' protocols with
157 minor adjustments. Briefly, the V3-V4 region was PCR amplified using universal primers
158 (Forward, 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN
159 GGC WGC AG-3′; Reverse, 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG
160 ACA GGA CTA CHV GGG TAT CTA ATC C-3′) (Klindworth et al., 2013), that contained
161 the Illumina adapter over- hang nucleotide sequences, using the NEBNext® Q5® Hot Start
162 HiFi DNA polymerase (New England Biolabs® Inc, Massachusetts, USA) and the following
163 thermocycling protocol: 98 °C/2 min, followed by 20 cycles of 98 °C/15 s, 63 °C/30 s, and
164 72 °C/30 s, and a final elongation step of 72 °C/5 min. Amplicons were purified using
165 AMPure XP PCR Purification beads (Beckman Coulter, Brea, California, USA), and the
166 NEBNext hot start high-fidelity DNA polymerase was used for the index PCR with Nextera
167 XT index primers (Illumina, San Diego, California, USA) according to the following
168 thermocycling protocol: 98 °C/30 s, 8 cycles of 98 °C/10 s, 65 °C/75 s and at 65 °C/5 min.
169 The indexed samples were purified using AMPure XP beads, quantified using the Qubit
170 Quant-iTTM dsDNA Broad-Range Assay Kit (Life Technologies, Carlsbad, California,
171 USA), and equal quantities from each sample pooled. The resulting pooled library was
172 quantified using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs®
173 Inc) and sequenced on an Illumina MiSeq platform using the v3 chemistry (301 bp paired-
174 end reads). ~~Raw sequence data have been deposited in the NCBI Sequence Read Archive~~

~~database under accession number XXXXXX [*The accession number will be inserted in the revised version of the manuscript*].~~

2.5. Bioinformatics analyses

Raw paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/), and reads were joined using the Quantitative Insights Into Microbial Ecology (QIIME) software suite (version 1.9.0) (Caporaso et al., 2010). Successfully joined sequences were quality filtered in QIIME using the 'usearch_qf' script with default settings. Then, sequences were clustered into Operational Taxonomic Units (OTUs) on the basis of similarity to known bacterial sequences available in the Greengenes database (v13.8; http://greengenes.secondgenome.com/; 97% sequence similarity cut-off) using the UCLUST software; sequences that could not be matched to references in the Greengenes database were clustered *de novo* based on pair-wise sequence identity (97% sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU. Then, representative sequences were assigned to taxonomy using the UCLUST software. OTUs assigned to sequences obtained from the no-DNA control samples, as well as singleton OTUs, were removed prior to downstream analysis. Normalisation was carried out by generating a subsampled OTU table by random sampling (without replacement) of the input OTU table using an implementation of the Mersenne twister algorithm (http://www.numpy.org). Cumulative-sum scaling (CSS) and log2 transformation were applied to account for the non-normal distribution of taxonomic counts data. Statistical analyses were executed using the Calypso software (cgenome.net/calypso/); samples were clustered using supervised Canonical Correlation Analysis including FEC (C*high*:C*low*) and time-point (D0, D2 and D14 p.t.) as explanatory variables. Differences in bacterial alpha diversity (Shannon diversity) between groups were

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evaluated using paired t-test or ANOVA, depending on number of groups for comparison. Beta diversity was calculated using weighted UniFrac distances and based on the matrices, differences in beta diversity were calculated using Permutational Analysis of Multivariate Dispersions (PERMDISP) through the 'betadisper' function (Anderson et al., 2006). Differences in the composition of the faecal microbiota between groups were assessed using the LEfSe (Linear discriminant analysis Effect Size) workflow (Segata et al., 2011), by assigning FEC/timepoint 'groupings' as comparison class. All statistical analyses were repeated on a sub-group of horses with FEC >200 e.p.g. (n=8) and 0 e.p.g. (n=7), hereafter referred to as 'C*200*' and 'C*0*', respectively.

3. Results

3.1 Prevalence of cyathostomin infection and group selection

Of 117 broodmares examined for cyathostomin infection, 36 matched the criteria outlined in section *2.2*. and were therefore enrolled in this study. Of these, 18 broodmares had an average FEC of ≥ 100 e.p.g. (range 100 - 418), and were thus enrolled as C*high* (Table 1). Amongst these, 8 were defined as C*200* based on average FEC of ≥ 200 e.p.g. (Table 1). Conversely, 18 broodmares with FEC of ≤ 10 e.p.g. (range 0 - 10) were enrolled as C*low* and, of these, 7 could be defined as C*0* (Table 1). While no known effect size estimates for changes in microbiota due to parasitism in horses are currently available, data from previous studies in other host:helminth systems (effect size: 1.5; cf. Giacomin et al., 2015, 2016) provided us with ~~suggest that the n = 18 animals in each C*high* and C*low* provided~~ 84% power to detect changes in gut microbial composition between groups (n=18 in each C*high* and C*low* group) using canonical analysies of principalle coordinates.

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Larval culture followed by species identification *via* RLB revealed infections by the following cyathostomin species: *Cyathostomum (Cya.) catinatum* (88%), *Cylicostephanus (Cys.) longibursatus* (71%), *Cylicocyclus (Cyc.) nassatus* (59%), *Coronocyclus coronatus* (41%), *Cyc. calicatus* (41%), *Cyc. radiatus* (29%), *Cys. goldi* (24%), *Cys. leptostomus* (18%), *Cya. pateratum* (18%), *Cyc. ashworthi* (18%) and ~~,~~ *Cyc. insigne* (12%). FEC analysis performed on samples collected at D2 and D14 p.t. showed ~~faecal egg count~~FEC reduction rates (FECR) of > 95% in all treated animals (Table 1).

3.2 Microbiota profiling

A total of 39,461,550 raw paired-end reads were generated from DNA faecal extracts of C*high* and C*low* broodmares and subjected to further processing. Following primer trimming, joining of paired-end reads, filtering of low-quality sequences and removal of 'contaminant' and singleton OTUs, a total of 8,077,490 (mean = 73,423 ± 3,610) high quality sequences were retained for further bioinformatics analysis (not shown). Raw and curated sequence data generated in this study are available from Mendeley Data at doi:10.17632/g7chkjrp8f.1. The rarefaction curves generated following in-silico subtraction of low-quality and contaminant sequences indicated that the vast majority of faecal bacterial communities were represented in the remaining sequence data, thus allowing us to undertake further analyses. These sequences were assigned to 95,286 OTUs and 15 bacterial phyla, respectively. The phyla Bacteroidetes (39.9%) and Firmicutes (34.0%) were predominant in all samples, followed by the phyla Verrucomicrobia (12.0%), Spirochaetes (3.9%), Fibrobacteres (2.4%), Cyanobacteria (1%), Proteobacteria (0.9%), Euryarcheota (0.4%), Tenericutes (0.4%), TM7 (0.3%), Actinobacteria (0.3%), Lentispheraerae (0.3%), Synergistetes (0.2%), WPS-2 (0.2%) and Planctomycetes (0.1%) (Fig. 1), while 3.4% of OTUs could not be assigned to any bacterial group. Predominant sub-taxa were Bacteroidia (class), Bacteroidales (order) and

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Bacteroidales (family) within the phylum Bacteroidetes, and Clostridia (class), Clostridiales (order) and Ruminococcae (family) within the Firmicutes (Fig. 1). Two samples, LVN1 and HS2, differed markedly in the relative proportions of the two most abundant phyla, Bacteroidetes and Firmicutes, when compared with samples from other broodmares (Fig. 1), likely indicating dysbiosis. Therefore, in order to reduce biases due to these potential 'outliers', these samples were excluded from further statistical analyses (Fig. 1).

Figure 1: Bar charts depicting the relative abundances of faecal bacterial phyla from broodmares with faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (= C*high*) and <10 e.p.g. (= C*low*), according to sampling time point (i.e. pre-anthelmintic treatment [D0], and 2 and 14 days post-treatment [D2 and D14, respectively], and in non-pregnant controls (NPC). Samples from broodmares with FEC >200 e.p.g. (*C200*) and 0 (*C0*) are shown in red.

3.3. Differences in microbial composition between Chigh and Clow, and pre- and post-anthelmintic treatment

Microbial community profiles of each sample were grouped by hierarchical clustering and ordinated by supervised CCA. Using t~~T~~hese methods, a ~~provided~~ significant association was observed between microbial composition and FEC (C*high versus* C*low*) (P = 0.003), while clustering according to time point pre- and post- anthelmintic treatment (D0 *versus* D14) did not reach statistical significance (P = 0.686) (Fig. 2a). CCA of C*200 versus* C*0* led to a clear separation according to FEC (P = 0.001) ~~(Fig. 2b)~~, whilst the effect of anthelmintic treatment remained insignificant (P = 0.811) (Fig. 2b).

Figure 2: The microbial composition of faecal samples ordered by supervised canonical correspondence analysis (CCA) from broodmares with (a) faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (= C*high*) and <10 e.p.g. (= C*low*), pre-anthelmintic treatment [D0] and at 14 days post-treatment [D14], (b) with FEC >200 e.p.g. (*C200*) and 0 (*C0*) at D0 and D14.

No significant differences in OTU alpha diversity (Shannon Index) were recorded between C*high* and C*low*, or between samples collected at D0, D2 and D14 p.t. (Fig.3 a,b,c). A trend towards increased alpha diversity in C*high versus* C*low* at all time-points was observed (P = 0.087) (Fig 3a). This trend was also observed when ~~consistent in~~ C*200* samples were compared to ~~versus~~ C*0* at ~~T~~ D0, despite smaller group sizes (P = 0.102) (Fig. 3c). No significant differences in beta diversity, as measured by PERMDISP, were observed between groups (Fig. 4).

Figure 3: Shannon diversity charts comparing faecal microbial alpha diversity of broodmares (a) with faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (= C*high*) and <10 e.p.g. (= C*low*) at all time points (i.e. pre-anthelmintic treatment [D0] and 2 and 14 days post-treatment [D2 and D14, respectively]), (b) C*high* and C*low* at D0 only, (c) with FEC >200 e.p.g. (C*200*) and 0 (C*0*) at D0 only, and (d) C*high* and ~~(according to sampling time point (i.e. D0, D2 and D14) and (~~e) C*low* at ~~according to sampling time point (i.e.~~ D0, D2 and D14~~)~~.

Figure 4: Permutational Analysis of Multivariate Dispersions (PERMDISP) plots comparing the faecal microbial beta diversity of broodmares (a) with faecal egg counts (FEC) >100 eggs per gram (e.p.g.) (C*high*) and <10 e.p.g. (C*low*) at all time points (i.e.

pre-anthelmintic treatment [D0] and 2 and 14 days post-treatment [D2 and D14, respectively]), (b) C*high* and C*low* at D0 only, (c) with FEC >200 e.p.g. (C*200*) and 0 (C*0*) at D0 only, and (d) C*high* and ~~according to sampling time point (i.e. D0, D2 and D14) and~~ (e) *Clow* ~~according to sampling time point (i.e.~~ at D0, D2 and D14~~)~~.

Differences in abundance of individual taxa at the phylum, class, order, family, genus and species level were detected between C*high* and C*low* samples, as well as between samples collected at D0, and D2 and D14 p.t. (Fig. 5). Samples from C*low* at D0 (pre-treatment) were characterised by an increased abundance of Methanobacteria (class), *Dehalobacterium* (genus) and unclassified *Dehalobacterium* and *Ruminococcus* (species) compared with samples from C*high* (Fig. 5a). The same taxa were increased in C*0* compared with C*200*, with the addition of methanogens of the family Methanocorpusculacaea belonging to order Methanomicrobiales, class Methanobacteria, phylum Euryarchaeota; order Endomicrobiales, phylum Elusimicrobia; Rickettsiales (order, family, genus, species); family Bacteroidaceae, genus BF311 and species RFN20 (Fig. 5b). The taxa GMD14H09 (order, family, genus, species) of the phylum Proteobacteria were increased in samples from C*200* compared with C*0* (Fig. 5b). Anthelmintic treatment in C*high* was accompanied by a decrease in the phylum TM7 at D14, when~~as~~ compared with pre-treatment samples (Fig. 5c). Additionally, the taxa *Adlercreutzia* and R445B were increased at D2 and D14, respectively, compared with D0 samples (Fig. 5c). In C*low*, treatment was also associated with an increase in R445B (family, genus, species) at D14 (Fig. 5d).

Figure 5: Linear discriminant analysis Effect Size (LEfSe) bar charts depicting differences in abundance of individual bacterial taxa at the phylum, class, order, family, genus and species level in faecal samples from broodmares (a) with faecal egg counts

(FEC) >100 eggs per gram (e.p.g.) (C*high*) and <10 e.p.g. (C*low*), (b) with FEC >200 e.p.g. (*C200*) and 0 (*C0*) at D0, and in (c) *Chigh* and (d) C*low* according to sampling time point (i.e. pre-anthelmintic treatment [D0] and 2 and 14 days post-treatment [D2 and D14, respectively]) ~~and (d) *Clow* according to sampling time point (i.e. D0, D2 and D14)~~.

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4. Discussion

This study is the first to report a significant association between numbers of cyathostomin eggs in faecal samples from UK Thoroughbreds and the composition of the ~~equine~~ host gut microbiota. A particularly significant shift in microbial profiles was observed when the faecal bacterial populations of a group of broodmares with FEC of >200 e.p.g. were compared with those with observed FEC of 0. These data are consistent with observations from published studies in both humans and other veterinary species, including rodent models of infection and disease (Lee et al., 2014; Holm et al., 2015; Houlden et al., 2015; McKenney et al., 2015; Duarte et al., 2016; ~~Holm et al., 2015; Houlden et al., 2015; Lee et al., 2014;~~ Li et al., 2016~~; McKenney et al., 2015~~). In addition, the administration of a routinely used anthelmintic (i.e. ivermectin) to both C*high* and C*low* resulted in further progressive changes of the microbial profiling of treated horses. While such changes ~~were statistically insignificant~~did not reach statistical significance when ~~tested~~ analysed using a multivariate model, this trend suggests that parasite-associated modifications in gut microbiota may be transient, and dependent on the presence of live infections, a hypothesis which requires thorough testing.

Overall, the bacterial phyla identified in this study were consistent between groups of animals enrolled in this study; this observation differs~~,~~ ~~which contrasts~~ from results from previous studies that had reported significant variability in faecal microbial profiling between horses, largely related to ~~differences~~ variations in diet and~~,~~ age, and presence of underlying diseases

(Costa et al., 2012; Daly et al., 2012; Steelman et al., 2012; O' Donnell et al., 2013; Dougal et al., 2014; Fernandes et al., 2014; ~~O' Donnell et al., 2013; Steelman et al., 2012;~~ Weese et al., 2014). Thus, our ~~observation~~ finding likely indicates that the impact of such confounding factors ~~linked to, e.g., diet, paddock, gender, age,~~ was successfully minimised by our study design, and that the recorded differences in microbial composition were indeed associated to parasite infections. ~~In contrast to previous studies in equids, b~~Bacteria belonging to the phylum Bacteroidetes were predominant in animals examined in our study; conversely, ~~,~~ ~~unlike~~ other investigations ~~that have~~ had reported Firmicutes as being the most prevalent phylum in the horse gut flora (Costa et al., 2012; Shepherd et al., 2012; Dougal et al., 2014; Fernandes et al., 2014; Weese et al., 2014; Costa et al., 2015a; Costa et al., 2015b; ~~Dougal et al., 2014; Fernandes et al., 2014;~~ Proudman et al., 2015~~; Shepherd et al., 2012; Weese et al., 2014~~). Dietary differences between horse cohorts enrolled in this and previous studies are likely to be responsible for this discrepancy (cf. Daly et al., 2012; Fernandes et al., 2014).

Overall, a trend towards increased microbial alpha diversity, i.e. the number of different OTUs in each sample ['richness'] and their relative abundance [= 'evenness'] (Tuomisto, 2010), was observed in samples from C*high* compared with those from C*low* at D0 (pre-anthelmintic treatment) and in C*200 versus C0*, although these differences did not reach statistical significance. Nevertheless, this observation is supported by the results of a number of previous studies in other host:helminth systems, in which the establishment of parasite infections was associated with an overall increase in alpha diversity of the gut microbiota (Broadhurst et al., 2012; Lee et al., 2014; Giacomin et al., 2015; Giacomin et al., 2016~~; Lee et al., 2014~~). Given that a number ~~many~~ of inflammatory GI and/or systemic diseases are accompanied by a reduced alpha diversity (Manichanh et al., 2006; Sepehri et al., 2007; Abrahamsson et al., 2012, 2014~~; Manichanh et al., 2006; Sepehri et al., 2007~~), the increase in

GI microbial diversity observed in the presence of helminth infections has been hypothesized to represent one of the possible mechanisms by which parasites suppress host inflammatory responses, thus ensuring their long-term survival in the host gut (Bancroft et al., 2012; Glendinning et al., 2014). Therefore, the trends towards increased alpha diversity observed in the faecal microbiota of horses moderately infected by cyathostomins may also result from an increase in gut homeostasis promoted by the parasite. Future studies evaluating the prevalence and incidence of equine inflammatory diseases (e.g. inflammatory bowel disease and recurrent airway obstruction) in the presence or absence of parasite infections could represent significant first steps in this area of research.

In addition to global microbial diversity, significant variations in the abundance~~s~~ of specific bacterial taxa were observed between groups. In particular, a higher abundance of microorganisms belonging to the class Methanomicrobia was observed in C*low* (D0) when compared to C*high*. This difference was exacerbated in C*200 versus* C*0*, with further significant increases in methanogens belonging to class Methanomicrobia recorded in C*0*, thus suggesting a negative correlation between methanogen abundance and FEC. Methanomicrobia belong to the phylum Euryarchaeota, kingdom Archaea and are phylogenetically distinct from bacteria and eukaryotes, although they retain the prokaryote 16S rRNA gene (Woese and Gupta, 1981; Winker and Woese, 1991~~; Woese and Gupta, 1981~~). Particularly in ruminants, the role of the Archaeal methanogens in the digestion of fibre has been well documented (St-Pierre et al., 2015). In equids, little is known of the functional diversity of methanogens; however, consistent with our findings, a recent study reported Methanomicrobiales as being predominant in the horse gut (Lwin and Matsui, 2014). The underlying mechanisms by which GI helminths may be promoting a reduction in populations of methanogens are yet unclear. Similarly to hypotheses formulated for other

host:helminth systems, cyathostomins may prevent expansion of methanogens directly, e.g. *via* their excretory secretory products, or indirectly *via* parasite-induced changes in mucosal immunity (reviewed by Peachey et al., 2017). Alternatively, a high abundance of methanogens prior to helminth infections may bias host immune responses against cyathostomins, thus resulting in the observed low (or absent) parasite burdens. Interestingly, some methanogens (i.e. *Methanosphaera stadtmanae*) have been shown to regulate Th17 responses in mice (Blais Lecours et al., 2011; Bernatchez et al., 2017~~; Blais Lecours et al., 2011~~); in turn, these responses have been linked to the ability of mice to clear experimental infections by *Heligmosomoides polygyrus* (Reynolds et al., 2014b~~a~~). Mechanistic studies ~~,~~ ~~e.g.~~ aimed to evaluate the effects of expanding populations of gut methanogens on host mucosal responses and, in turn, GI helminth establishment, may help elucidating these interactions.

An increased abundance of Methanomicrobia in C*low* and C*0* may also be linked ~~the result of~~ to other environmental factors that are simultaneously responsible for the low FEC observed. An example ~~of such a factor~~ is represented by the horse grazing behaviour; indeed, it is known that ~~certain~~ some individuals within a herd favour less nutritional swards of grass in order to avoid faecal contamination (Hutchings et al., 2000). In turn, as animal faeces often act as fertilisers ~~and~~, as a result, individuals favouring nutritious grass are exposed to higher numbers of infective larvae. Grazing different swards of grass may also impact on dietary fibre levels, and thus on gut methanogen populations, as observed in ruminants (McAllister et al., 1996). In horses, dietary factors have also been associated with changes in abundance of Methanomicrobia; for example, *Methanocorpusculum* archaea were observed at a median of 17.7% in horses fed a forage-grain diet, and at a median of 31.9% in horses maintained on pasture (Fernandes et al., 2014). Differences in grazing behaviour between individuals may

also be accountable for the increased abundance of bacteria of the phylum Elusimicrobia in C*0* versus C*200* as these taxa are primarily a component of termite hind-gut microbiota (~~Mikaelyan et al., 2017;~~ Gómez and González-Megías, 2007; van Klink et al., 2015; Mikaelyan et al., 2017). Experimental cyathostomin infections of stabled horses may eliminate the effect of grazing behaviour on gut microbial profiles, although ethical concerns may prevent the execution of such a study in the future.

In contrast to uninfected horses, the faecal microbial profiles of C*200* were characterised by an increased abundance of GMD14H09, phylum Proteobacteria, class Deltaproteobacteria. Increases in Proteobacteria abundance have repeatedly been reported in association with helminth infections, e.g. in mice infected by *Trichuris muris* and *H. polygyrus*, pigs infected by *T. suis*, and rabbits infected by *Trichostrongylus retortaeformis* (~~Cattadori et al., 2016; Holm et al., 2015;~~ Li et al., 2012; Holm et al., 2015; Cattadori et al., 2016). Proteobacteria are known increase in the presence of ~~to be enriched with~~ GI inflammation ~~and can lead to sustained microbial dysbiosis~~ (Shin et al., 2015); hence, the expansion of populations of Proteobacteria in the faecal microbiota of horses with higher infection burdens ~~is unsurprising~~may be indicative of an inflammatory status of the intestinal tract of these horses at the time of sampling.

One of the objectives of this study was to assess the impact of anthelmintic treatment on the faecal microbial profiling of cyathostomin-infected horses. In particular, ivermectin administration to C*high* was followed by a significant decrease in populations of the phylum TM7 at D14. Since the relative abundance of TM7 remained ~~were~~ unchanged following ivermectin administration in C*low*, it is tempting to speculate that a mutualistic association may exist between TM7 and cyathostomins, whereby each promote establishment of the

other, similarly to the mutual relationship ~~that~~ described for Lactobacillacaeae and *H. polygyrus* (Reynolds et al., 2014a~~b~~). Bacteria belonging to the phylum TM7 are obligate epibionts of *Actinomyces* spp. (He et al., 2015), and are thus uncultivable. While TM7 have~~s~~ not previously been linked to GI helminth infections, this phylum of bacteria has been associated with mucosal inflammatory disease in humans (Kuehbacher et al., 2008). Interestingly, ~~isolates from~~ TM7 isolates have been shown to repress the induction of TNF-α production in macrophages infected by *Actinomyces odontolyticus*, thus suggesting a potential immune suppressive activity (He et al., 2015); ~~therefore~~hence, TM7 may promote the establishment of cyathostomins by suppressing effective anti-parasite immune responses. Furthermore, an increase of~~in~~ the taxa *Adlercreutzia* (phylum actinobacteria) and R445B (phylum Lentisphearae) was observed in C*high* at D2 and D14, respectively. The latter was also increased in C*low* at D14, suggesting that this change was unrelated to cyathostomin removal. Bacteria of the genus *Adlercreutzia* produce the metabolite equol (Maruo et al., 2008), a known anti-inflammatory and vasodilator (Blay et al., 2010). ~~Hence~~Thus, it could be hypothesised that increases in populations of *Adlercreutzia* and its metabolites following ivermectin administration might contribute to the emergence of hypobiotic larval stages of cyathostomins (~~,~~ which is known to occur post-anthelmintic treatment; ~~(~~Lyons et al., 2000), via ~~suppress~~the suppression of~~ing~~ effective mucosal immune response~~ity~~. This hypothesis requires testing in controlled mechanistic experiments.

FEC are often utilised as proxy of parasite infection burdens; however, several investigations have confuted this practice, as weak correlations have been detected between FEC and parasite burdens in horses with >500 e.p.g. of faeces (Nielsen et al., 2010). While the FEC cut-offs used in this study are indicative of differing infection burdens between groups, any inference on the relationships between number of worms in the horse intestine and gut microbial profiling must be taken with caution. Ethical considerations prevent us from

performing post-mortem total worm counts on experimentally infected horses; nevertheless, in the future, it may be possible to establish unequivocal relationships between cyathostomin infection burdens (including encysted larvae) and gut microbial profiling from samples collected in abattoir.

Clearly, a complex network of host-parasite interactions, as well as environmental factors, contribute to the findings reported in this study, and thus further work is needed to disentangle the causality of these relationships. However, one key question that needs addressing is whether differences in host immunity may be associated with significant changes in gut microbial composition (and *vice versa*) and, if such is the case~~o~~, whether the horse gut microbiota could be manipulated to improve resistance to helminth infection. Indeed, previous investigations in cattle and mice have reported that host genes encoding for antimicrobial proteins are up-regulated in the mucosae of animals ~~which are bred for~~ resistant~~ce~~ to helminth infection (D'Elia et al., 2009; Li et al., 2015). In addition, dietary supplementation with both pro- (Bautista-Garfias et al., 1999; Bautista-Garfias et al., 2001; Martinez-Gomez et al., 2009; Martinez-Gomez et al., 2011; Oliveira-Sequeira et al., 2014; El Temsahy et al., 2015~~; Martinez-Gomez et al., 2011; Martinez-Gomez et al., 2009; Oliveira-Sequeira et al., 2014~~) and pre-biotics (~~Jensen et al., 2011;~~ Petkevicius et al., 2003; Petkevicius et al., 2004; Thomsen et al., 2005; Petkevicius et al., 2007; Jensen et al., 2011~~; Thomsen et al., 2005~~), has led to significant reductions in worm burdens in murine and swine helminth infection models, thus indicating that alterations to the bacterial flora in the host GI tract may bias host immune responses against ~~helminth infection~~parasites. ~~Consequently,~~ ~~f~~Further characterisation of equine host mucosal responses and GI microbiota, in the presence or absence of helminth infection and accompanied by total enumeration of infecting parasites,

is a key area of future research, as it may lead to the identification of microbial factors linked to host susceptibility.

5. Conclusion

Cyathostomin infection in horses was associated with global shifts in faecal microbial composition and diversity, in accordance with previous studies in other host: helminth systems, as well as significant changes in specific populations of gut bacteria. Such changes predominantly involved 'minor' phyla, thus suggesting that the equine 'core gut microbiota' remains unaltered in the presence of burdens of cyathostomins such as those observed in this study. Our findings also support the hypothesis that selected bacterial taxa, and/or their metabolites, may play roles in biasing the host immune response either for, e.g. TM7, or against, e.g. Methanomicrobia, cyathostomin infection in horses. These data pave the way for future mechanistic studies aimed to identify microbial factors linked to host susceptibility, and to ~~alter~~ manipulate the GI microbiota of horses (e.g. via dietary or probiotic interventions), in order to improve resistance to cyathostomins.

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Table 1: ~~Results of f~~Faecal egg counts (FEC) ~~analysis~~ recorded from C*high* (FEC >100 eggs per gram, e.p.g.) and C*low* (FEC <100 e.p.g.) broodmares enrolled in the study, as well as of non-pregnant controls (NPC), over ~~selected into two groups: 1) with mean FEC over~~ three consecutive samplings performed ~~of >100 eggs per gram (e.p.g.) (C*high*), and 2) with mean FEC over three consecutive samplings of < 10 e.p.g. (C*low*),~~ ~~both~~ pre-anthelmintic treatment [D0], as well as ~~and~~ at 2 and 14 days post-treatment [D2, D14]. ~~Animals~~ Horses with FEC of > 200 e.p.g. (C*200*) and 0 e.p.g. (C*0*), are indicated in bold.

Group ~~i.d.~~	~~*Horse*~~ *Animal i.d.*	*Age (yrs)*	*Tapeworm FEC (e.p.g.)*	*Ascarid FEC (e.p.g.)*	*Mean consecutive strongyle FEC (e.p.g.) (±SE) D0*	*FEC D2 (e.p.g.)*	*FEC D14 (e.p.g.)*
Chigh							
	MA	6	0	0	123 (±19)	0	0
	CT	5	0	0	130 (±14)	0	0
	LE	10	0	0	113 (±17)	0	0
	PB	5	0	0	171 (±15)	0	0
	SC	4	0	0	101 (±8)	2 (±2)	0
	MSJ	6	0	0	100 (±6)	0	0
	WD	7	0	0	128 (±16)	0	0
	NS	8	0	0	120 (±11)	1 (±1)	0
	HY	7	0	0	150 (±14)	0	0
	RM	4	0	0	139 (±49)	0	0
	HS	**6**	**0**	**0**	**200 (±39)**	**1 (±1)**	**1 (±1)**
	HT	**6**	**0**	**0**	**228 (±22)**	**0**	**0**
	NO	**6**	**0**	**0**	**271 (±17)**	**0**	**1 (±1)**
	QM	**4**	**0**	**0**	**418 (±112)**	**0**	**0**
	SB	**8**	**0**	**0**	**206 (±37)**	**1 (±1)**	**1 (±1)**
	TC	**4**	**0**	**0**	**235 (±12)**	**0**	**1 (±1)**
	MQ	**6**	**0**	**0**	**228 (±22)**	**0**	**0**
	VR	**4**	**0**	**0**	**279 (±61)**	**1 (±1)**	**0**
Clow							
	GL	4	0	0	10 (±2)	0	0
	DWD	8	0	0	0.3 (± 0.3)	0	0
	LVN	4	0	0	5 (±1)	0	0
	BB	7	0	0	1 (±1)	0	4 (±1)
	LF	5	0	0	10 (±2)	0	0
	IR	7	0	0	3 (±1)	0	0
	DDR	8	0	0	1 (±1)	0	0
	LAL	5	0	0	0.3 (±0.3)	0	0
	PT	7	0	0	0.3 (±0.3)	0	0
	PP	6	0	0	2 (±1)	0	0
	MG	5	0	0	0.5 (±0.3)	0	0
	SY	**8**	**0**	**0**	**0**	**0**	**0**
	BX	**16**	**0**	**0**	**0**	**0**	**0**
	MR	**10**	**0**	**0**	**0**	**0**	**0**

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	DD		**0**	**0**	**0**	**0**	**0**
	ED	**8**	**0**	**0**	**0**	**0**	**0**
	EP	**5**	**0**	**0**	**0**	**0**	**0**
	SWC	**8**	**0**	**0**	**0**	**0**	**0**
~~*Non-pregnant control*~~*NPC*							
	BET	8	0	0	412 (±45)	-	-
	BL	12	0	0	22 (±1)	-	-
	ST	10	0	0	0	-	-

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Figure 1

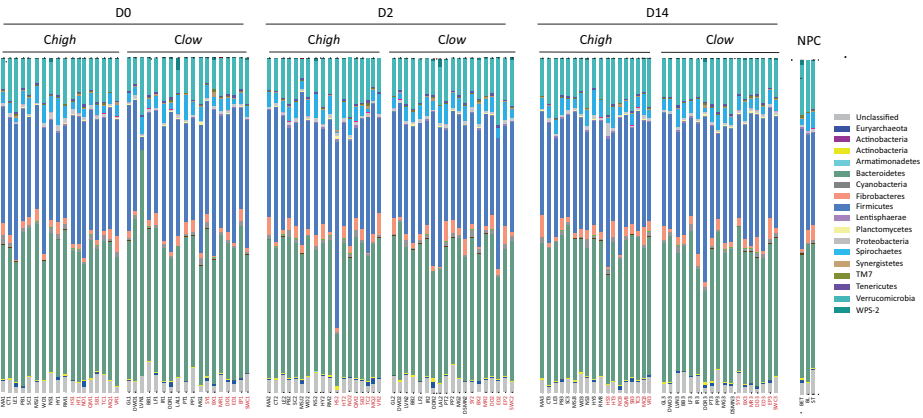

D0
D2
D14
NPC
Chigh
Clow
Unclassified
Euryarchaeota
Actinobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Cyanobacteria
Fibrobacteres
Firmicutes
Lentisphaerae
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
TM7
Tenericutes
Verrucomicrobia
WPS-2

Figure 2

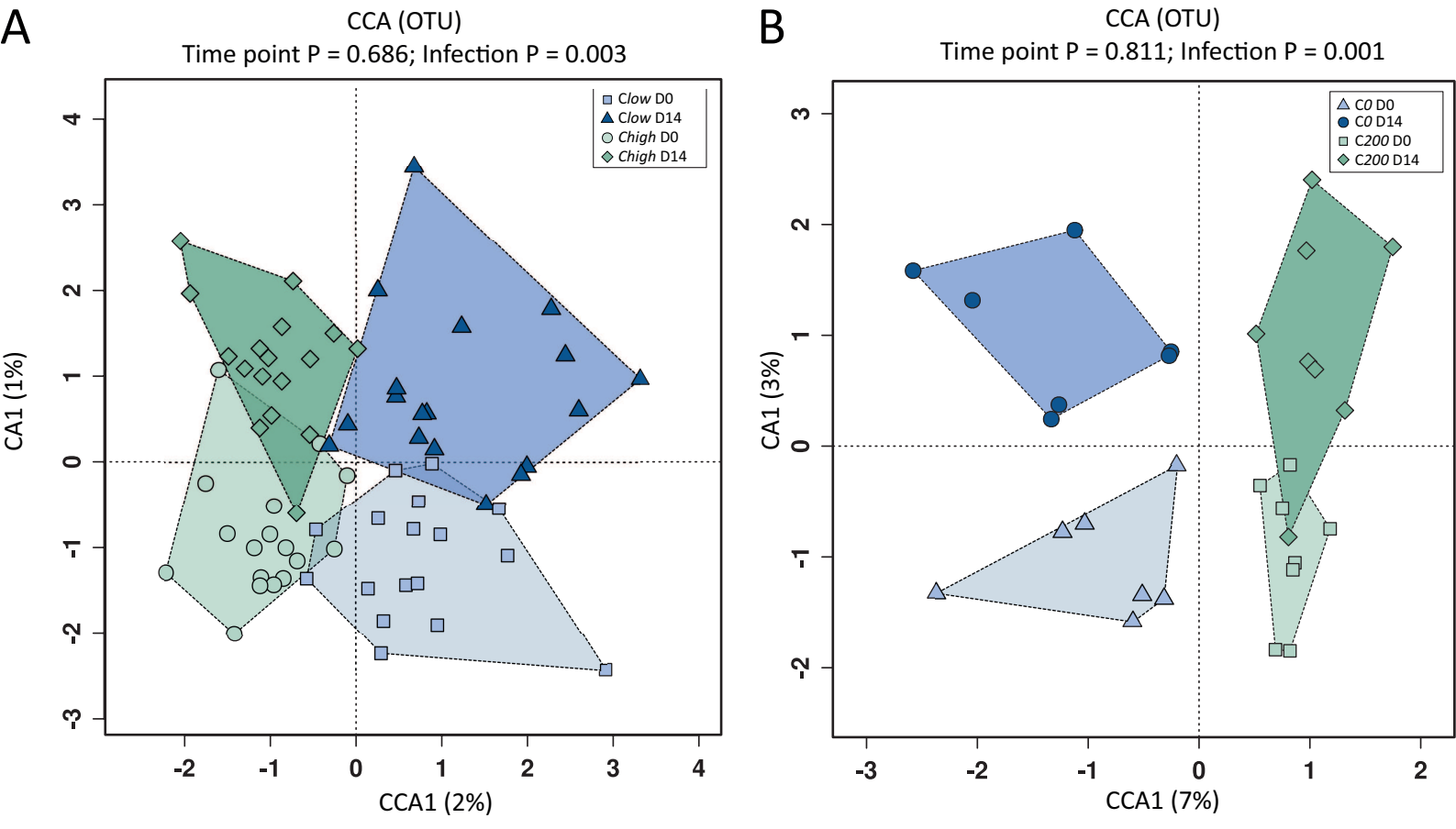

A
CCA (OTU)
Time point P = 0.686; Infection P = 0.003
Clow D0
Clow D14
Chigh D0
Chigh D14
CA1 (1%)
CCA1 (2%)
B
CCA (OTU)
Time point P = 0.811; Infection P = 0.001
C0 D0
C0 D14
C200 D0
C200 D14
CA1 (3%)
CCA1 (7%)

Figure 3

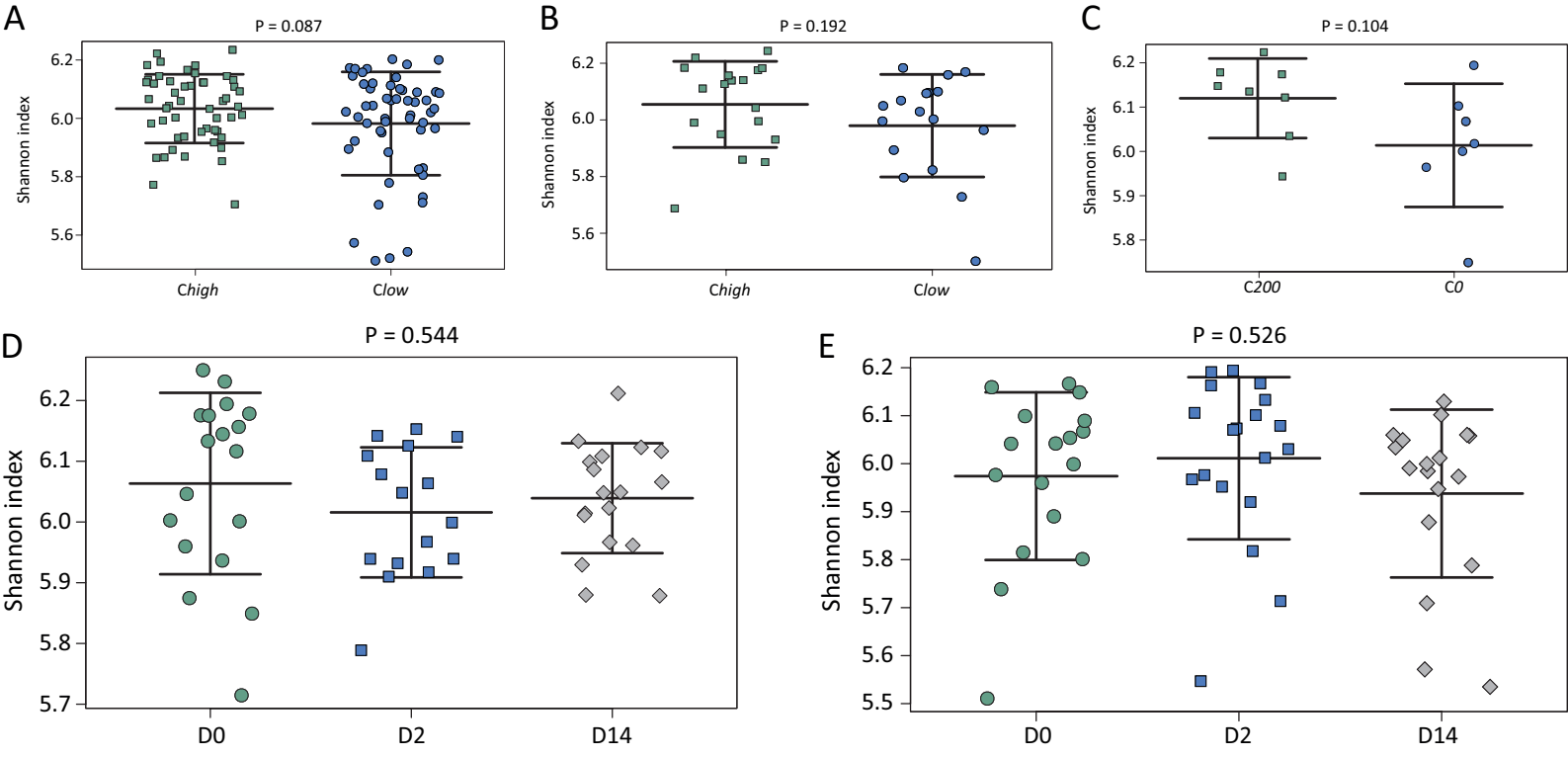

A
P = 0.087
Shannon index
6.2
6.0
5.8
5.6
Chigh
Clow
B
P = 0.192
Shannon index
6.2
6.0
5.8
5.6
Chigh
Clow
C
P = 0.104
Shannon index
6.2
6.1
6.0
5.9
5.8
C200
C0
D
P = 0.544
Shannon index
6.2
6.1
6.0
5.9
5.8
5.7
D0
D2
D14
E
P = 0.526
Shannon index
6.2
6.1
6.0
5.9
5.8
5.7
5.6
5.5
D0
D2
D14

Figure 4

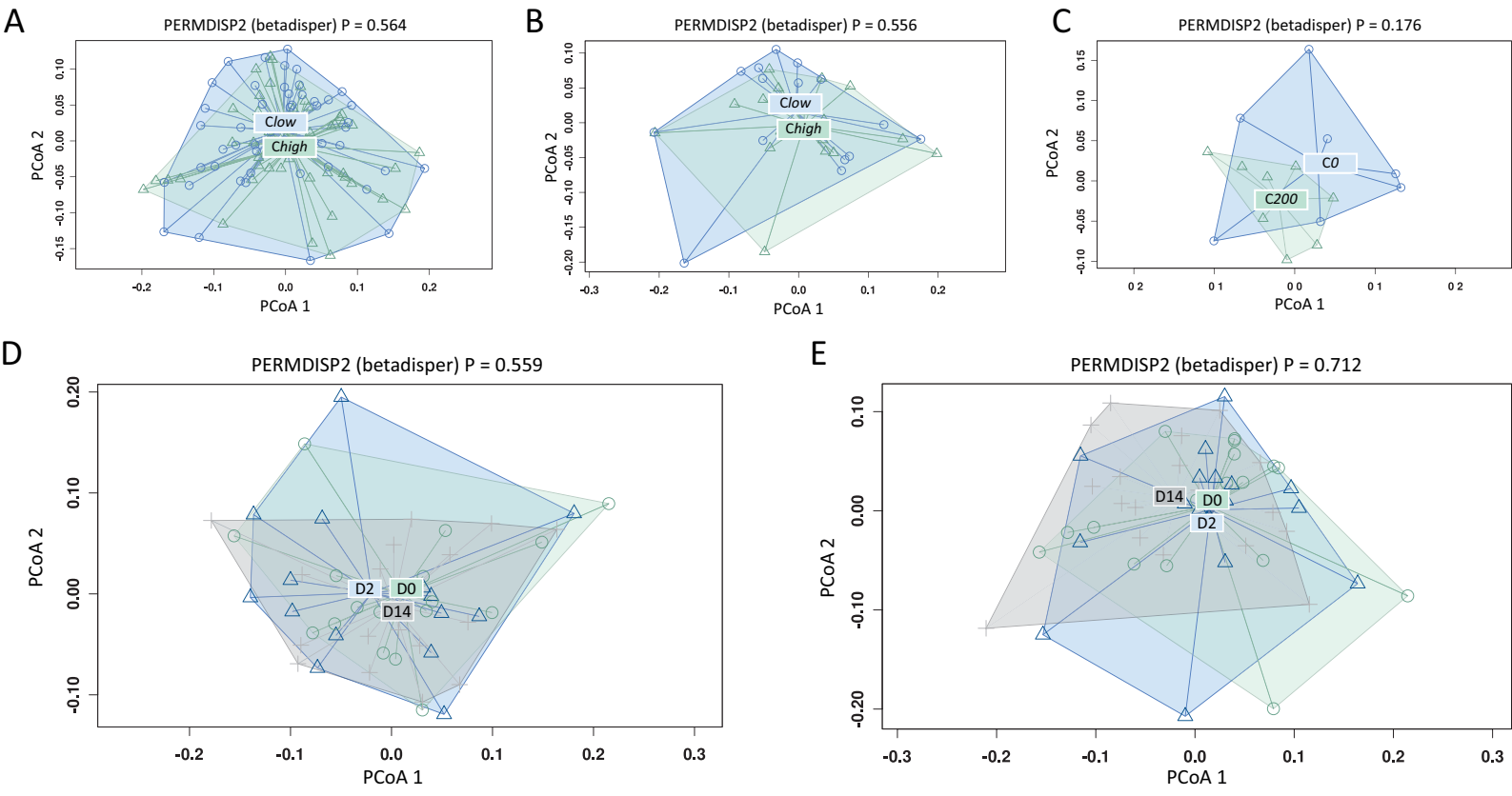
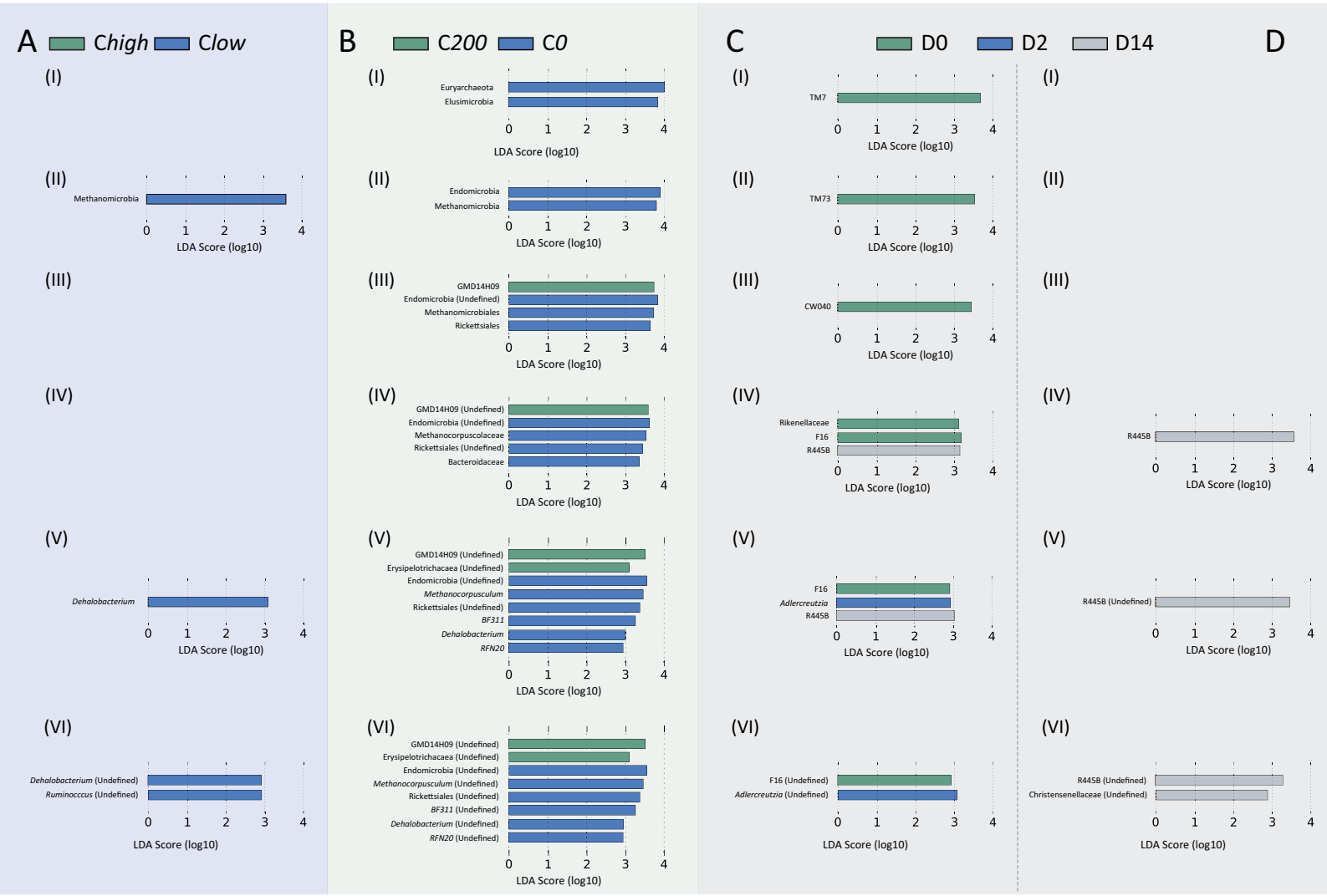

A
PERMDISP2 (betadisper) P = 0.564
Clow
Chigh
PCoA 2
PCoA 1
B
PERMDISP2 (betadisper) P = 0.556
Clow
Chigh
PCoA 2
PCoA 1
C
PERMDISP2 (betadisper) P = 0.176
C0
C200
PCoA 2
PCoA 1
D
PERMDISP2 (betadisper) P = 0.559
D2
D0
D14
PCoA 2
PCoA 1
E
PERMDISP2 (betadisper) P = 0.712
D14
D0
D2
PCoA 2
PCoA 1

Figure 5


A
Chigh
Clow
(I)
(II)
Methanomicrobia
(III)
(IV)
(V)
Dehalobacterium
(VI)
Dehalobacterium (Undefined)
Ruminocccus (Undefined)
LDA Score (log10)
B
C200
C0
(I)
Euryarchaeota
Elusimicrobia
(II)
Endomicrobia
Methanomicrobia
(III)
GMD14H09
Endomicrobia (Undefined)
Methanomicrobiales
Rickettsiales
(IV)
GMD14H09 (Undefined)
Endomicrobia (Undefined)
Methanocorpuscolaceae
Rickettsiales (Undefined)
Bacteroidaceae
(V)
GMD14H09 (Undefined)
Erysipelotrichacaea (Undefined)
Endomicrobia (Undefined)
Methanocorpusculum
Rickettsiales (Undefined)
BF311
Dehalobacterium
RFN20
(VI)
GMD14H09 (Undefined)
Erysipelotrichacaea (Undefined)
Endomicrobia (Undefined)
Methanocorpusculum (Undefined)
Rickettsiales (Undefined)
BF311 (Undefined)
Dehalobacterium (Undefined)
RFN20 (Undefined)
LDA Score (log10)
C
D0
D2
D14
(I)
TM7
(II)
TM73
(III)
CW040
(IV)
Rikenellaceae
F16
R445B
(V)
F16
Adlercreutzia
R445B
(VI)
F16 (Undefined)
Adlercreutzia (Undefined)
LDA Score (log10)
D
(I)
(II)
(III)
(IV)
R445B
(V)
R445B (Undefined)
(VI)
R445B (Undefined)
Christensenellaceae (Undefined)
LDA Score (log10)